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H. Heimpel, Ulm · D. Huhn, München
C. Mueller-Eckhardt, Gießen
G. Ruhenstroth-Bauer, München

Modern Trends in Human Leukemia V

New Results
in Clinical and Biological Research
Including Pediatric Oncology

Organized on behalf of the Deutsche Gesellschaft für Hämatologie und Onkologie. Wilsede, June 21–23, 1982

Wilsede Joint Meeting on Pediatric Oncology II
Hamburg, June 25/26, 1982

Edited by
R. Neth, R. C. Gallo, M. F. Greaves,
M. A. S. Moore, and K. Winkler

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Dr. Rolf Neth, Universitaets-Krankenhaus Eppendorf, Medizinische Klinik, Abteilung fuer klinische Chemie, Martinistraße 52, 2000 Hamburg 20, Federal Republic of Germany

Dr. Robert C. Gallo, National Cancer Institute, Laboratory of Tumor Cell Biology, Bethesda, MD 20014, USA

Dr. Melvyn F. Greaves, Imperial Cancer Research Fund Laboratories, P.O. Box 123, London WC2 A3PK, Great Britain

Dr. Malcolm A. S. Moore, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA

Dr. Kurt Winkler, Universitaets-Kinderklinik, Abteilung fuer Haematologie und Onkologie, Martinistraße 52, 2000 Hamburg 20, Federal Republic of Germany

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Scientific Organisation of the Sessions

New Strategies in Leukemia Diagnostic and Therapy

Heimpel, Hermann, Zentrum fuer Innere Medizin und Kinderheilkunde der Universitaet Ulm, Steinhoevelstrasse 9, 7900 Ulm, Federal Republic of Germany
Hoffbrand, A. Viktor, Dept. of Haematology, Royal Free Hospital, Rond Street, Hampstead, London NW32QG, Great Britain

Mechanism of Malignant Transformation
Virology

Gallo, Robert, National Cancer Institute, Bldg. 37, Room 6BO4, Bethesda, MA 20014, USA
Hausen, Harald zur, Institut fuer Virologie im Zentrum fuer Hygiene, Hermann-Herder-Strasse 11, 7800 Freiburg, Federal Republic of Germany

Cellbiology

Greaves, Melvyn F., Imperial Cancer Research Fund Laboratories, P.O. Box 123, London WC2A3PX, Great Britain
Moore, Malcolm A.S., Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA

Immunological Aspects in Malignancy

Cooper, Max, University of Alabama, School of Medicine, Comprehensive Cancer Center, Birmingham, AL 35294, USA
Mitchison, N. Avrion, University College London, Tumour Immunology Unit, Gower Street, London WC1E6BT, Great Britain

Wilsede Joint Meeting on Pediatric Oncology II

Kabisch, Hartmut, Universitaetskinderklinik, Abteilung für Haematologie und Onkologie, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany
Winkler, Kurt, Universitaetskinderklinik, Abteilung für Haematologie und Onkologie, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany

Local Organisation

Mannweiler, Klaus, Heinrich-Pette-Institut fuer experimentelle Virologie und Immunologie an der Universitaet Hamburg, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany
Neth, Rolf, Universitaetskrankenhaus Eppendorf, Medizinische Klinik, Abteilung fuer klinische Chemie, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany

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[3] Presented in the Wilsede Joint Meeting on Pediatric Oncology II

Participants of the Meeting

Anders, Fritz, Genetisches Institut der Universitaet, Heinrich-Buff-Ring 58–62, 6300 Giessen, Federal Republic of Germany

Bauer, Georg, Institut fuer Virologie im Zentrum für Hygiene, Hermann-Herder-Strasse 11, 7800 Freiburg, Federal Republic of Germany

Bell, Richard, Medical Oncology University Hospital, 75 East Newton Street, Boston, MA 02062, USA

Bernhard, Silke, Dahlem-Konferenzen, Wallotstrasse 19, 1000 Berlin 33, Federal Republic of Germany

Bister, Klaus, Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 63–73, 1000 Berlin 33, Federal Republic of Germany

Blattner, William A., Family Studies Section, Environmental Epidemiology Branch, National Cancer Institute, Landow Building, Rm. 4C18, 7910 Woodmont Avenue, Bethesda, MD 20205, USA

Boiron, Michel, Institut de Recherches sur les Leucemies et les Maladies du Sang, Université Paris VII, Hôpital Saint-Louis, 2 Place du Docteur-Fournier, 75475 Paris Cedex 10, France

Boniver, Jacques, Institut de Pathologie B 23, Laboratoire d'Anatomie, Pathologique, 4000 Liège, Belgium

Bornkamm, Georg W., Institut fuer Virologie im Zentrum fuer Hygiene, Hermann-Herder-Strasse 11, 7800 Freiburg, Federal Republic of Germany

Burgess, Antony W., Ludwid Institute for Cancer Research, Melbourne Tumour Biology Unit, Royal Melbourne Hospital, Victoria 3050, Australia

Burny, Arsène, Faculté des Sciences, Laboratoire de Chimie Biologique, 67 Rue des Chevauc, 1640 Rhode-St-Genèse, Belgium

Chen, Po-min, Veterans General Hospital, Dept. of Hematology, Taipei, Taiwan 112, Republic of China

Chessels, Judith M., The Hospital for Sick Children, Dept. of Haematology and Oncology, Great Ormond Street, London WC1N3JH, Great Britain

Chieco-Bianchi, Luigi, University of Padova, Dept. of Oncology, Via A. Gabelli 61, 351000 Padova, Italy

Cooper, Max D., UAB, Comprehensive Cancer Center, Cellular Immunobiology Unit, Dept. of Pediatrics and Microbiology, Birmingham, AL 35294, USA

Creutzig, Ursula, Universitaets-Kinderklinik, Robert-Koch-Strasse 31, 4400 Muenster, Federal Republic of Germany

Dalla Favera, Riccardo, National Cancer Institute, Building 37, Rm. 6 CO4, Bethesda, MD 20014, USA

Dautry, François, MIT, Center for Cancer Research, 77 Massachusetts Avenue, Boston, MA 02139, USA

Deinhardt, Friedrich, Max-v.-Pettenkofer-Institut, Pettenkoferstrasse 9a, 8000 Muenchen 2, Federal Republic of Germany

Diehl, Volker, Medizinische Hochschule Hannover, Zentrum Innere Medizin und Dermatologie, Karl-Wiechert-Allee 9, 3000 Hannover 61, Federal Republic of Germany

Duesberg, Peter, Dept. of Molecular Biology, University of California, Wendell M. Stanley Hall, Berkeley, CA 94720, USA

Ehrlich, Rachel, George S. Wise Faculty of Life Sciences, Dept. of Microbiology, Tel Aviv, Israel

Elstner, Elena, Universitaetsklinik fuer Innere Medizin, Charite, Schumannstrasse 20/21, 1040 Berlin, German Democratic Republic

Erfle, Volker, Gesellschaft fuer Strahlen- und Umweltschaeden, Institut für Biologie, 8042 Neuherberg, Federal Republic of Germany

Essex, Max, Harvard School of Public Health, Dept. of Microbiology, 665 Huntington Avenue, Boston, MA 02115, USA

Ferrari, Sergio, Instituto di Clinica Medica, Policlinico, Via del Pozzo 71, 41100 Modena, Italy

Fischinger, Peter J., National Cancer Institute, Building 31, Rm. 11A56, Bethesda, MD 20014, USA

Freudenstein, Christa, Institut fuer Virusforschung, DKFZ, Im Neuenheimer Feld 280, 6900 Heidelberg, Federal Republic of Germany

Galili, Naomi, Hadassah Medical School, Dept. of Virology, Jerusalem, Israel

Gallo, Robert C., National Cancer Institute, Building 37, Rm. 6BO4, Bethesda, MD 20014, USA

Galton, David A. G., MRC, Leukaemia Unit, Hammersmith Hospital, Ducane Road, London E12OHS, Great Britain

Gershon, Richard K., Yale University, Dept. of Pathology, 310 Cedar Street, New Haven, CT 06510, USA

Gianni, Alessandro M., Instituto di Clinica Medica I, Padiglione Granelli, Via Francesso Sforza 35, 20122 Milano, Italy

Goldblum, Natan, Hadassah Medical School, Dept. of Virology, Jerusalem, Israel

Grandori, Carla, Instituto Suoeriore di Sanita, Laboratorio Pathologia non Infettiva, Viale Regina Elena 299, 00161 Rome, Italy

Greaves, Melvyn F., Imperial Cancer Research Fund Laboratories, Membrane Immunology Laboratory, Lincoln's Inn Fields, London WC2A3PX, Great Britain

Haseltine, William A., Sidney Farber Cancer Institute, The Jimmy Fund, 45 Binney Street, Boston, MA 02115, USA

Hausen, Harald zur, Institut fuer Virologie im Zentrum fuer Hygiene, Hermann-Herder-Strasse 11, 7800 Freiburg, Federal Republic of Germany

Hehlmann, Rüdiger, Medizinische Poliklinik der Universitaet Muenchen, Pettenkoferstrasse 8 a, 8000 Muenchen 2, Federal Republic of Germany

Heimpel, Hermann, Zentrum fuer Innere Medizin und Kinderheilkunde der Universitaet Ulm, Steinhoevelstrasse 9, 7900 Ulm, Federal Republic of Germany

Helm, Klaus von der, Max-v.-Pettenkofer-Institut, Pettenkoferstrasse 9a, 8000 Muenchen 2, Federal Republic of Germany

Henderson, Edward S., Roswell Park Memorial Institute, Medicine A, 666 Elm Street, Buffalo, NY 14263, USA

Hoelzer, Dieter, Zentrum fuer Innere Medizin und Kinderheilkunde der Universitaet, Steinhoevelstrasse 9, 7900 Ulm, Federal Republic of Germany

Hoffbrand, A. Victor, The Royal Free Hospital, Dept. of Haematology, Pond Street, Hampstead, London NW32QG, Great Britain

Hofschneider, Peter-Hans, Max-Planck-Institut fuer Biochemie, 8033 Martinsried, Federal Republic of Germany

Hossfeld, Dieter Kurt, Medizinische Universitaetsklinik, Abteilung Onkologie/Haematologie, Martinistrasse 52, 2000 Hamburg 20

Izaguirre, Carlos, Imperial Cancer Research Fund Latoratories, St. Bartholomew's Hospital, West Smithfield, London EC1A7BE, Great Britain

Jacquemin, Paul, Institut Ludwig de Recherches sur le Cancer, Avenue Hippocrate 74, UCL 7459, 1200 Brussels, Belgium

Jaenisch, Rudolf, Heinrich Pette-Institut fuer experimentelle Virologie und Immunologie, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany

Kaplan, Henry S., Stanford University Medical Center, Dept. of Radiology, Cancer Biology Research Laboratory, Stanford, CA 94305, USA

Kersey, John H., Dept. of Laboratory Medicine, 420 Delaware Street S.E., Box 609 – Mayo, Minneapolis, MN 55455, USA

Kisseljov, Fjodor L., Cancer Research Center, Kashirskoie Shosse 6, Moscow 115478, USSR

Konze-Thomas, Beate, Deutsche Forschungsgemeinschaft, Kennedyallee 40, 5300 Bonn-Bad Godesberg, Federal Republic of Germany

Kramer, Gisela, Clayton Foundation Biochemical Institute, Dept. of Chemistry, Austin, TX 78712, USA

Kurth, Reinhard, Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstrasse 37–39, 7400 Tuebingen, Federal Republic of Germany

L'Age-Stehr, Johanna, Robert-Koch-Institut des Bundesgesundheitsamtes, Abteilung Virologie, Nordufer 20, 1000 Berlin 65, Federal Republic of Germany

Lane, Mary-Ann, DANA 940A, Laboratory of Molecular Carcinogenesis, Sidney Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA

Lau, Barbara, Institut fuer Haematologie, Abteilung Experimentelle Haematologie, Landwehrstrasse 61, 8000 Muenchen 2, Federal Republic of Germany

Lennert, Karl, Institut fuer Pathologie, Abteilung Allgemeine Pathologie und Pathologische Anatomie, Hospitalstrasse 42, 2300 Kiel, Federal Republic of Germany

Levy, Jean-Paul, Laboratoire Immunologie et Virologie des Tumeurs, Hôpital Cochin, Groupe INSERM U152, 27 Rue du Foubourg Saint-Jacques, 75674 Paris Cedex 14, France

Lister, T. Andrew, Imperial Cancer Research Fund Laboratories, Dept. of Medical Oncology, St. Bartholomew's Hospital, West Smithfield, London EC1A7BE, Great Britain

Mannweiler, Klaus, Heinrich-Pette-Institut fuer experimentelle Virologie und Immunologie, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany

Mertelsmann, Roland, Memorial Sloan-Kettering, Cancer Center, 1275 York Avenue, New York, NY 10021, USA

Metcalf, Donald, The Walter and Eliza Hall Institute of Medical Research, Cance Research Unit, Royal Melbourne Hospital, Victoria 3050, Australia

Milleck, Jürgen, Zentralinstitut für Krebsforschung, Lindenberger Weg 80, 1115 Berlin-Buch, German Democratic Republic

Miller, Denis R., Memorial Sloan-Kettering, Cancer Center, 1275 York Avenue, New York, NY 10021, USA

Minowada, Jun, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263, USA

Mitchison, N. Avrion, University College London, Tumour Immunology Unit, Gower Street, London WC1E6BT, Great Britain

Mölling, Karin, Max-Planck-Institut fuer Molekulare Genetik, Ihnestrasse 63–73, 1000 Berlin 33, Federal Republic of Germany

Moore, Malcolm A. S., Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA

Munk, Klaus, Institut fuer Virusforschung, DKFZ, Im Neuenheimer Feld 280, 6900 Heidelberg, Federal Republic of Germany

Myers, Richard M., Harvard University, Cambridge, MA 02248, USA

Neth, Rolf-Dietmar, Abteilung fuer Klinische Chemie der Medizinischen Klinik, Universitaetskrankenhaus Eppendorf, Martinistrasse 62, 2000 Hamburg 20, Federal Republic of Germany

Nicola, Nicos A., The Walter and Eliza Hall Institute of Medical Research, Cancer Research Unit, Royal Melbourne Hospital, Victoria 3050, Australia

Olsson, Inge L., Research Department 2, E-blocket, University Hospital, 221 85 Lund, Sweden

Papamichail, Michail, Hellenic Anticancer Institut, 171, Alexandras Ave, Athen, Greece

Papas, Takis S., Carcinogenesis Regulation Section, Laboratory of Molecular Oncology, NIH, Building 41, Suite 400, Bethesda, MD 20014, USA

Peschle, Cesare, Instituto Superiore di Sanità, Laboratorio Patologia non Infettiva, Viale Regina Elena 299, 00161 Rome, Italy

Pimentel, Enrique, Instituto de Medicina Experimentas, Apartado Postal 50587, Caracas 1050-A, Venezuela

Pinkel, Donald, Sunny and Isadore Familian Children's Hospital, City of Hope National Medical Center, 1500 East Duarte Road, Duarte, CA 91010, USA

Ploegh, Hidde L., Institut fuer Genetik der Universitaet zu Koeln, Weyertal 121, 5000 Koeln 41, Federal Republic of Germany

Pragnell, Ian B., The Beatson Institute for Cancer Research, Wolfson Laboratory for Molecular Pathology, Garscube Estate, Switchback Road, Bearsden, Glasgow G611BD, Great Britain

Ramot, Bracha, Chaim Sheba Medical Center, Institute of Hematology, Tel Hashomer, Israel

Reitz, Marvin, National Cancer Institute, Building 37, Rm. 6CO9, Bethesda, MD 20014, USA

Riethmüller, Gert, Institut fuer Immunologie, Schillerstrasse 42, 8000 Muenchen 2, Federal Republic of Germany

Rigby, Peter W. J., Imperial College of Science and Technology, Dept. of Biochemistry, London SW72AZ, Great Britain

Rodt, Hans, Institut fuer Haematologie, Abteilung Immunologie, Landwehrstrasse 61, 8000 Muenchen 2, Federal Republic of Germany

Rohatiner, Ama, Imperial Cancer Research Fund Laboratories, Dept. of Medical Oncology, St. Bartholomew's Hospital, West Smithfield, London EC1A7BE, Great Britain

Sarin, Prem S., National Cancer Institute, Building 37, Rm. 6AO9, Bethesda, MD 20014, USA

Sarngadharan, Mangallasserie, National Cancer Institute, Building 37, Rm. 6AO9, Bethesda, MD 20014, USA

Schreiber, Hans, La Rabida Children's Hospital and Research Center, Dept. of Pathology, East 65 Street at Lake Michigan, Chicago, IL 60649, USA

Schubert, Johannes, St.-Joseph-Hospital, Wiener Strasse 1, 2050 Bremerhaven, Federal Republic of Germany

Simon, Markus, Max-Planck-Institut fuer Immunbiologie, Stuebeweg 51, 7800 Freiburg-Zaehringen, Federal Republic of Germany

Singer, Jack W., Veterans Administration Medical Center, 4435 Beacon Avenue South, Seattle, WA 98108, USA

Slevin, Maurice, Imperial Cancer Research Fund Laboratories Dept. of Medical Oncology, St. Bartholomew's Hospital, West Smithfield, London EC1A7BE, Great Britain

Stein, Harald, Institut fuer Pathologie, Abteilung Allgemeine Pathologie und Pathologische Anatomie, Hospitalstrasse 42, 2300 Kiel, Federal Republic of Germany

Tax, Wil, St. Radboudziekenhuis, Klinik voor Inwendige Ziekten, Afdeling Nephrologie, Geert Grooteplein zuid 8, 6500 HB Nijmegen, The Netherlands

Terhorst, Cox, Sidney Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA

Thomas, E. Donnall, The Fred Hutchinson Cancer Research Center, Division of Oncology, Dept. of Medicine, 1124 Columbia Street, Seattle, WA 98104, USA

Thorpe, Philip E., Imperial Cancer Research Fund Laboratories, Holburn, London WC2A3PX, Great Britain

Torelli, Giuseppe, Instituto di Clinica Medica, Policlinico, Via del Pozzo 71, 41100 Modena, Italy

Torelli, Umberto, Instituto di Clinica Medica, Policlinico, Via del Pozzo 71, 41100 Modena, Italy

Uchańska-Ziegler, Barbara, Medizinische Klinik, Abteilung Innere Medizin II, Immunologie-Labor, Otfried Mueller-Strasse, 7400 Tuebingen

Vande Woude, George F., NIH, Building 41, Rm. 100, Laboratory of Molecular Oncology, Bethesda, MD 20205, USA

Vondelich, Laida, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London WC2A3PX, Great Britain

Watt, Suzanne, Dept. of Pathology, Division of Immunology, Addenbrooke's Hospital, Hills Road, Cambridge CB22QQ, Great Britain

Weinstein, Howard J., Sidney Farber Cancer Institute, Dept. of Pediatric Oncology, 44 Binney Street, Boston, MA 02115, USA

Weiss, Elisabeth, National Institute for Medical Research, The Ridgeway, London NW71AA, Great Britain

Weiss, Robin A., Institute of Cancer Research, Royal Cancer Center, Chester Beatty Research Institute, Fulham Road, London SW36JB, Great Britain

Wigzell, Hans, Biomedicinska Centrum, Avdelingen foer Immunologi, 75123 Uppsala, Sweden

Wilson, Elaine L., University of Cape Town, Dept. of Clinical Science and Immunology, Observatory, 7925 Cape, South Africa

Wolf, Hans, Max-v.-Pettenkofer-Institut, Arbeitsgruppe Molekulare Virologie, Pettenkoferstrasse 9a, 8000 Muenchen 2, Federal Republic of Germany

Wong-Staal, Flossie, National Cancer Institute, Building 37, Bethesda, MD 20014, USA

Young, Brian, The Beatson Institute for Cancer Research, Garscube Estate Switchback Road, Bearsden, Glasgow G611BD, Great Britain

Zintl, Felix, Universitaets-Kinderklinik, Kochstrasse 2, 6900 Jena, German Democratic Republic

Wilsede Scholarship Holders

(Granted by the Deutsche Krebshilfe, Leukemia Research Fund Great Britain, and the Leukemia Society of America)

Andreesen, Reinhard, Medizinische Universitaetsklinik, Abteilung Innere Medizin I, Hugstetter Strasse 55, 7800 Freiburg, Federal Republic of Germany

Augener, Wiprecht, Medizinische Klinik, Haematologische Abteilung, Hufelandstrasse 55, 4300 Essen, Federal Republic of Germany

Becker, Thomas, Kinder-Poliklinik, Feulgenstrasse 12, 6300 Giessen, Federal Republic of Germany

Geoffrey, Brown, Dept. of Immunology, Vincent Drive, Birmingham B152TJ, Great Britain

Crockard, Alistair D., Dept. of Haematology, Royal Victoria Hospital, Belfast BT126BA, Northern Ireland

Dölken, Gottfried, Medizinische Universitaetsklinik, Hugstetter Strasse 55, 7800 Freiburg, Federal Republic of Germany

Drexler, H. Günter, Zentrum fuer Kinderheilkunde der Universitaet Ulm, Prittwitzstrasse 43, 7900 Ulm, Federal Republic of Germany

Erhardt, Johannes, Institut für Immunologie, Schillerstrasse 42, 8000 Muenchen 2, Federal Republic of Germany

Flood, Patrick M., Yale University, Dept. of Pathology, Brady Memorial Laboratory, FMB 202, 210 Cedar Street, New Haven, CT 06510, USA

Francis, Gillian, The Royal Free Hospital, Dept. of Haematology, Pond Street, London NW32QG, Great Britain

Ganser, Arnold, Abteilung fuer Klinische Physiologie und Arbeitsmedizin, Oberer Eselsberg, 7900 Ulm, Federal Republic of Germany

Gassmann, Winfried, II. Medizinische Klinik, Metzstrasse 53/57, 2300 Kiel, Federal Republic of Germany

Haas, Wolfgang, Pathologisches Institut, Joseph-Stelzmann-Strasse 9, 5000 Koeln 41, Federal Republic of Germany

Haworth, Catherine, Paterson Laboratories, Christie Hospital, Holt Radium Institute, Withington, Manchester M209BX, Great Britain

Heckmayr, Marlene, II. Medizinische Klinik, Abteilung Onkologie und Haematologie, Universitaetskrankenhaus Eppendorf, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany

Hiddemann, Wolfgang, Medizinische Klinik Abteilung Innere Medizin A, Westring 3, 4400 Muenster, Federal Republic of Germany

Hirsch, Friedrich, Medizinische Universitaetsklinik, Hugstetter Strasse 55, 7800 Freiburg, Federal Republic of Germany

Höfer, Paul, Institut fuer Immunologie, Schillerstrasse 42, 8000 Muenchen 2, Federal Republic of Germany

Jäger, Gundula, Institut fuer Haematologie, Landwehrstrasse 61, 8000 Muenchen 2, Federal Republic of Germany

Jendis, Jörg, Max-Planck-Institut fuer Biochemie, Abteilung Hofschneider, 8033 Martinsried, Federal Republic of Germany

Kanz, Lothar, Medizinische Universitaetsklinik, Abteilung Haematologie und Onkologie, Hugstetter Strasse 55, 7800 Freiburg, Federal Republic of Germany

Mills, K., University College London, Dept. of Haematology, Gower Street, London WC1E6AU, Great Britain
Müller, Dorith, Institut und Poliklinik fuer Klinische Immunologie und Rheumatologie, Krankenhausstrasse 12, 8520 Erlangen, Federal Republic of Germany
Mullins, Jim, Harward School of Public Health, Dept. of Microbiology, 66 Huntington Avenue, Boston, MA 02115, USA
Murray, Lesley, Imperial Cancer Research Fund Laboratory, Dept. of Medical Oncology, St. Bartholomew's Hospital, West Smithfield, London EC1A7BE, Great Britain
Nobis, Peter, Abteilung Molekularbiologie, Physiologisch-Chemisches Institut, Grindelallee 117, 2000 Hamburg 13, Federal Republic of Germany
Porzsolt, Franz, Zentrum fuer Innere Medizin der Universitaet Ulm, Steinhoevelstrasse 9, 7900 Ulm, Federal Republic of Germany
Ritter, Jörg, Kinderklinik, Abteilung Haematologie und Onkologie, Robert-Koch-Strasse, 4400 Muenster, Federal Republic of Germany
Rübsamen, Helga, Paul-Ehrlich-Institut, Bundesamt fuer Sera und Impfstoffe, Paul-Ehrlich-Strasse 42–44, 6000 Frankfurt, Federal Republic of Germany
Schlimok, Günter, II. Medizinische Klinik, Langemarckstrasse 11, 8900 Augsburg, Federal Republic of Germany
Scott, Martin, Stanford University Medical Center, Dept. of Radiology, Cancer Biology Research Laboratory, Stanford, CA 94305, USA
Sieber, Gerhard, Universitaetsklinikum Steglitz, Abteilung Innere Medizin, Haematologie und Onkologie, Hindenburgdamm 30, 1000 Berlin 45, Federal Republic of Germany
Sieff, Colin, The Hospital for Sick Children, Dept. of Haematology, Great Ormond Street, London WC1N3JH, Great Britain
Siegert, Wolfgang, Medizinische Klinik III, Klinikum Großhadern, 8000 Muenchen 70, Federal Republic of Germany
Strobel, Eva-Susanne, Husserlstrasse 4, 7800 Freiburg, Federal Republic of Germany
Stünkel, Klaus, Institut für Haematologie, Abteilung Immunologie, Landwehrstrasse 61, 8000 Muenchen 2, Federal Republic of Germany
Thöne, Ingo, Universitaets-Kinderklinik, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany
Welte, Karl, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA
Wilkie, N., The Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Bearsden, Glasgow G611BD, Great Britain
Ziegler, Andreas, Medizinische Universitaetsklinik, Labor fuer Immunologe, 7400 Tuebingen, Federal Republic of Germany
Zigler, Hans-Werner L., Institut für Immunologie, Schillerstrasse 42, 8000 Muenchen 2, Federal Republic of Germany
Ziemiecki, Andrew, Institut fuer Virologie, Frankfurter Strasse 107, 6300 Giessen, Federal Republic of Germany

Preface

Wir fühlen, daß selbst, wenn alle möglichen wissenschaftlichen Fragen beantwortet sind, unsere Lebensprobleme noch garnicht berührt sind.

Ludwig Wittgenstein,
Tractus logico-philosophicus (1921),
Annalen der Naturphilosophie

In June 1982, physicans and scientists met in Wilsede for the fifth time to discuss origin and treatment of leukemia.

Lectures and discussions of the meeting closely demonstrated that leukemia can be considered a perfect model for the studies of biology and treatment of cancer in general. The Wilsede meeting has confirmed that cancer is a multifactorial disease in which oncogenes, regulator proteins, the immunsystem, viruses and chemical noxes play important roles. The development of new revolutionary techniques in biological research yielded important insights into the causes of leukemia which are also promising to develop new ways for the treatment of cancer patients.

I am indepted to the chairmen organizing a stimulating and exciting program which included all the present and future aspects of human leukemia.

I am grateful to all participating scientists, the supporting stuff and the Wilsede people for making this meeting both scientifically excellent as well as humanly warm and unforgettable. Last but not least, it must be emphazised that the "Verein Naturschutzpark Lüneburger Heide" and Dr. h.c. Alfred Toepfer are responsible for saving this peaceful place for us.

Hamburg, February 1982 Rolf Neth

Coffee break and lectures in "De Emmenhoff".
Middle left, Wellcome of Dr. h.c. Alfred Toepfer
Down left, Melvin Greaves starting his Stohlman lecture
Down right, Donall Thomas giving his Stohlman lecture

Personal and scientific discussions in Wilsede, June 1982

(Fotos: Regina Völz, Cetric Parkin)

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Frederick Stohlman Jr.
Memorial Lecture

Wilsede, June 21 1978

Memorial Tribute to Dr. Frederick Stohlman Presented by William C. Moloney

Gallo, Robert C.: Cellular and Virological Studies Directed to the Pathogenesis of the Human Myelogenous Leukemias

Pinkel, Donald: Treatment of Childhood Acute Lymphocytic Leukemia

Wilsede, June 18 1980

Klein, George: The Relative Role of Viral Transformation and Specific Cytogenetic Changes in the Development of Murine and Human Lymphomas

Kaplan, Henry S.: On the Biology and Immunology of Hodgkin's Disease

Wilsede, June 21 1982

Greaves, Melvyn F.: Immunobiology of Lymphoid Malignancy

Thomas, E. Donnall: Bone Marrow Transplantation in Leukemia

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Edited by Neth, Gallo, Greaves, Moore, Winkler

Immunobiology of Lymphoid Malignancy

M. F. Greaves

A. Introduction

The study of 'membrane markers' in human leukaemia has now been in progress for a decade. Starting from the initial observation of L. Borella and colleagues at St. Judes on the sub-types of ALL [1] a wealth of data has accumulated particularly over the past few years with the introduction of monoclonal antibodies. Now is perhaps a good time to appraise the impact of these efforts and the implications for future research on leukaemia.

As Seligmann, Kersey, myself and others have emphasised on many occasions, the single most fruitful product of this activity has been the appreciation of how the cellular heterogeneity of lymphoid leukaemia and lymphoma mirrors stages of normal differentiation. This clearly arises as a consequence of three salient features of haemopoietic malignancy: the restricted or clonal origin [2], the imposition of maturation arrest, and the broad conservation or fidelity of a qualitatively normal phenotype [3].

The immunological and enzymatic definition of leukaemic cell phenotypes in relation to their normal counterparts has direct relevance to clinical problems of differential diagnosis, patient monitoring and variable prognosis [4]. Immunological features of ALL subgroups for example are linked to known prognostic features (e.g. high white cell count in T-ALL) and not surprisingly, therefore, show a strong correlation with the outcome of chemotherapy [1, 4–6]. Combinations of markers (e.g. cell surface antigens and nuclear terminal transferase [7]) offer the possibility of monitoring leukaemia and detecting residual, minimal or re-emerging extramedullary disease (i.e. CNS or testis).

The application of a panel of monoclonal antibodies has been routinely applied in my own laboratory for a national immunodiagnostic service over a number of years. It is difficult to determine precisely how useful such a service is; however, I estimate that the phenotypic data are essential in something like 15% of cases and are useful or supporting in many more (perhaps the majority). All of this is clear and undisputed; I would rather emphasise the broader and more substantial impact which I believe these studies should have.

Firstly, they provide a rational, biological framework for attempts to improve the efficacy of therapy either by more selective or 'tailored' allocation of particular regimes to defined leukaemic subgroups or by exploiting the biological information to design new or more radical strategies, e.g. monoclonal antibody elimination of leukaemic cells, selective enzyme inhibition. Secondly, they provide an essential framework for pursuing the molecular basis of haemopoietic malignancy. Since cellular oncogenes (or their viral homologues) are probably limited in number and have some important function in regulating normal differentiation and/or proliferation, it is of some importance to search for these genes and the expression and function of their products in the context of particular leukaemic subtypes and their normal counterparts; this is indeed already happening (see papers by F. Wong-Staal and M. A. Lane in this volume).

Some of the above points can be emphasised with reference to the biology of ALL.

B. Heterogeneity and Origins of ALL

Acute lymphoblastic leukaemia can be dissected in a number of subgroups with exclusive, composite phenotypes, which correlate with prognosis [4]. More recently, the use of monoclonal antibodies and immunoglobulin gene probes and the study of maturation induction in vitro has further elucidated the nature of ALL cells. It is now clear that ALL consists of two broad subtypes, both of which originate in lymphocyte progenitors (Table 1); one is 'pre-T' or equivalent to thymic precursors of mature T cells; the other, more common, variant is 'pre-B' or equivalent to B-cell progenitors and precursors in bone marrow. Within these two categories subtypes can be defined which broadly reflect sequential stages of maturation within the 'early' compartments of these two distinct cell lineages [8–10).

Table 1. Biological features of two ALL subtypes

	B precursor-ALL	T precursor-ALL
Dominant phenotype[a]	TdT^+ $HLA\text{-}DR^+$ T^- B^+ $cALL^+$ $Hex\text{-}I^+$	TdT^+ $HLA\text{-}DR^-$ T^+ B^- $cALL^-$ $Hex\text{-}I^-$
Ig genes	$\mu \pm \kappa/\lambda$ re-arr.[b]	No or minimal re-arr.
Growth fraction	Low	High
Karyotype	Hyperdiploidy common	Pseudodiploidy common
Likely cellular origin	Bone marrow B-lineage progenitor or stem cell	Marrow or thymic (subcapsular) T-lineage progenitor or stem cell
Diagnostic subtypes	Common ALL Pre-B ALL Null-ALL	T-ALL
Alternative diagnoses	AUL Ph^{1+} ALL NHL (rare)	T-NHL

[a] Serologically defined cell surface antigens or intracellular enzymes – terminal deoxynucleotidyl transferase and hexosaminidase isoenzyme I (plus charge variants of other acid lysosomal hydrolases: [42])

[b] Ig genes (e.g. V, D, J, μ heavy chain) re-arranged from germ line configuration [41]

Detailed studies on the antigenic phenotypes of these leukaemias provide no evidence for qualitatively aberrant gene expression or for a progenitor cell shared by and exclusive to the T and B lineages. Thus, ALL cells do not express glycophorin [11] or other restricted non-lymphoid markers; neither do they show concurrent expression on single cells or within a single leukaemic clone of markers unique to T and B cells. The 'pre-T' and 'pre-B' categories are also consistent features and although individual markers may change in relapse [12] there is no shift between these two subtypes during malignant progression in individual patients [3]. Normal counterparts of the ALL subtypes with qualitatively similar phenotypes (excluding karyotype) can be found in bone marrow [9, 13] and thymus [8, 10].

It is of some interest to note that whereas malignancies of lymphocyte precursors occur predominantly in children and young patients, malignancies of mature lymphoid cells (leukaemia, lymphoma, myeloma) are almost exclusively adult diseases [13a]; one interpretation of this correlation and the similarly striking age associations of other cancers (e.g. neural tumours versus epithelial carcinomas) is that they are a reflection of cell populations (stem cells?) at risk through proliferative demand at various stages of early development or during prolonged function (and turnover) in adult life.

The simplest interpretation of this descriptive data is therefore that ALL can originate in progenitor cells of either the T- or B-cell lineage and invariably suffers from the imposition of maturation arrest with the conservation of phenotype 'appropriate' for the particular stage of differentition in which the leukaemic cells become frozen or stabilised. Whilst I believe this general conclusion to be manifestly

correct there are some relevant and important qualifications that should be appreciated:

1. The phenotypes observed are not identical for every leukaemic blast cell of an individual patient. Phenotypic categorisation reflects the dominant phenotype, but in practice some diversity can always be detected either with respect to quantity (e.g. antigen density) or in what appears to be quality. Figure 1 illustrates one such case, in which one-third of the leukaemic cells have a different but clearly related phenotype to the other two-thirds. The interpretation favoured for this intraclonal diversity is that it reflects in large part the variable stringency of maturation arrest, i.e. all cells do not appear to be stopped in their tracks at precisely the same developmental position. Superimposed upon this maturational control there is also some phenotypic diversity which is linked to cell proliferation, e.g. expression of the monoclonal antibody defined receptor for transferrin [14].

2. Detailed scrutiny of ALL phenotypes in relation to their supposed normal counterparts suggests that they are probably not perfect replicas; an analogy with the minimally deviated hepatomas of Potter [15] may be appropriate. The "abnormalities" concern some apparent deletions, such as lack of expression of the E rosette "receptor" or TdT when the remainder of the composite phenotype dictates that they be present or what can best be described as asynchronies of gene expression, i.e. combinations of markers which are normally sequentially expressed in maturation, such as TdT and high-density HLA-ABC in T-ALL [10, 16], TdT and μ chains in pre-B ALL [17, 40].

3. Leukaemias with an identical (non-chromosomal) phenotype to ALL can arise in the pluripotential stem cell. As reviewed elsewhere [18] approximately one-third of Ph^1-positive CGL in blast crisis have the common ALL or B-cell progenitor phenotype which includes monoclonal antibody defined antigens, selective enzyme expression and also re-arranged Ig genes (Mulgaard, Gould and Greaves, unpublished observations). Some adult patients can present with Ph^1 ALL without a clinically evident chronic phase CGL but may after

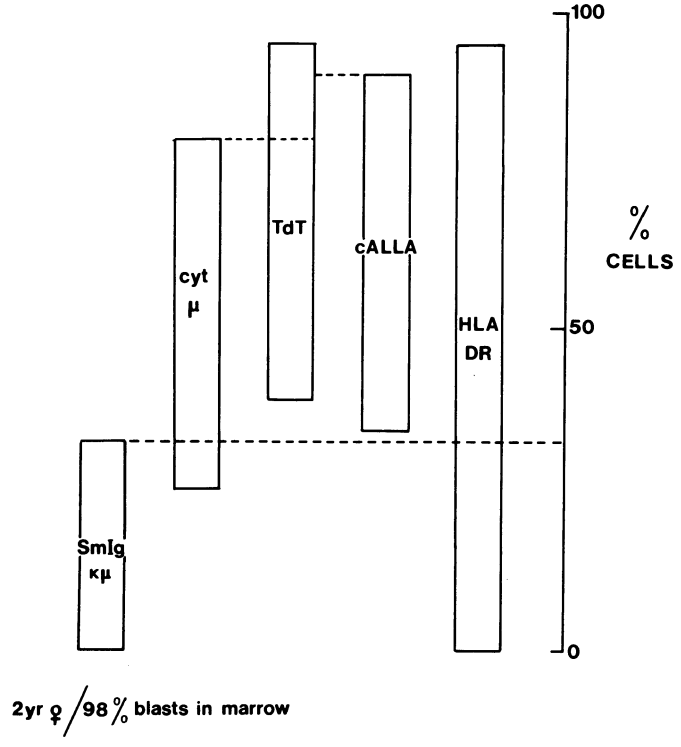

Fig. 1. Variable position of "maturation arrest" in cALL. Bone marrow lymphoblasts were stained with various combinations of reagents to analyse phenotypic diversity, e.g. anti-DR, anti-cALL, anti-μ or anti-Ig (κ/λ) in combination with TdT; anti-κ in combination with anti-DR or anti-cALL

therapy revert to CGL [18]. It is important to note that whereas B-cell progenitor ALL (e.g. common ALL) is curable with chemotherapy, blast crises manifest in this cellular compartment are not, although as expected they may achieve short-term remissions with steroids [19]. This sharp distinction provides an excellent example of the importance of "target cell" biology for understanding clinical outcome and developing appropriate alternative therapeutic strategies (e.g. marrow transplants for Ph^1-positive leukaemia).

4. ALL of either B or T progenitor type may not be diagnosed haematologically as ALL. Thus the majority of those rare (~5%) acute leukaemias which haematologists consider to be acute undifferentiated leukaemia are usually identifiable as ALL subtypes or more rarely as immature myeloid cells [4, 20]. Paediatric cases diagnosed as non-Hodgkin lymphoma may also belong or at least be very closely related to the two major subtypes of ALL. Conversely, not all cases diagnosed as ALL may be bona fide ALL. Thus, B-ALL is probably a misnomer; this relatively mature B-cell leukaemia probably represents a rapidly disseminating lymphoma [4, 21]. Rare cases of newborn acute leukaemia diagnosed as ALL may in fact be 'cryptic' erythroleukaemias as assessed by studies with monoclonal antibodies including anti-glycophorin [11, 22].

5. The maturation arrest imposed in ALL may be reversible, at least partially in vitro. Thus, some T-ALL cell lines can be induced by phorbol ester (TPA) to irreversibly modulate their composite phenotype from that of an immature or thymic variety to that of a mature T-cell subset [23, 24]. We and others have also been able to modulate the expression of TdT and cell surface antigen in B-cell progenitor ALL, although in our experience Ig synthesis cannot be induced in Ig^- ALL despite the presence of re-arranged μ chain genes. Our interpretation of this is that in leukaemia and in normal B-cell differentiation these recombinational genetic events are inefficient, with most clones failing to achieve a productive or functional re-arrangement.

The observation that maturation arrest in ALL is reversible as demonstrated previously with other leukaemias (e.g. Friend virus erythroleukaemia and myeloid leukaemia in rodents, avian erythroleukaemia and in some human leukaemic cell lines, e.g. HL-60, K562) carries the important corollary that maturation arrest, a central "lesion" in acute leukaemia, is a regulatory defect which, although having a genetic, inheritable basis, is reversible in its phenotypic consequences.

C. Is the Conservation of Phenotype Telling Us Anything Interesting About Leukaemic Cells?

It could be argued that since malignancy involves rare genetic events, it is to be expected that these will not have catastrophic effects on a cell's pattern of gene expression and that the broad fidelity of phenotype observed in ALL is (a) just what we would expect, and (b) boring and of no relevance or even downright misleading with respect to the central issue of what distinguishes a leukaemic cell from normal. Furthermore, it can always be that the 'critical' gene products in leukaemia are not those which we rather arbitrarily elect to study (so far) and that a more appropriate screen would reveal distinct, qualitative and consistent differences between leukaemic cells and their normal counterparts. These are not unreasonable views and I am surprised that they are not made more often.

I have favoured a different view initially because it was more interesting and subsequently because I believe it is supported by data. That is that the expression of qualitatively normal phenotype or pattern of gene expression is an integral and essential feature of most if not all leukaemias and other malignancies. Qualitative abnormalities (e.g. new or lost antigens, altered glycolipids, altered drug recognition) may occur and indeed have some selective advantage with malignant progression and treatment; however, they need not be considered as essential components of the malignant state. In the context of ALL, therefore, and as suggested some years ago [25, 26] a qualitatively normal lymphoid progenitor cell phenotype *which is normally only transiently expressed on proliferating cells* is quite compatible with leukaemic

Table 2. Structure, genetics and function of ALL-associated membrane proteins identified by monoclonal antibodies

	Monoclonal antibody (ref.)					
	J-5 [31]	BA-2 [34]	DA-2 [39]	OKT9 [14, 37]	OKT10 [37]	OKT11 [38]
Structural features:						
General	Single polypeptide ~ 100K [32, 33]	Single polypeptide ~ 24K	Two non-covalently linked polypeptides 28K (β), 33K (α) + intracellular 30K pp (HLA-DR)	Two s-s linked polypeptides ~ 90K [14, 37]	Single polypeptide ~ 40K [37, 38]	Single polypeptide ~ 40K
Glycosylated	+	±	+	+	+	+
pI	5.2 [33]	7.3 [35]	α: 5, β: 7[a] [36]	5.0 [28]		
Peripheral (p) or integral (I), transmembrane (t)	p	p	I.t.	I.t.	I	
Genetics: (chromosomal control)	?	12[b]	6	3 [29]	4[b]	
Function:	?	?	Cell interactions	Transferrin receptor [14] NK 'target'? [30]	?	Receptor for sheep erythrocytes[c] [38]

[a] Mean value; multiple spots observed with variable positions reflecting allelic polymorphism
[b] Katz, Povey and Greaves, unpublished observations
[c] Natural, physiological function unknown

cell behaviour and only requires that the genetic change provoking clonal selection effectively uncouples proliferation from maturation.

This view accords with recent molecular studies which reveal the central role of normal genes (*c-onc*) or their inserted viral (*v-onc*) homologues which may facilitate clonal advantage via amplification or excessive promotion ([27] and various papers in this volume). There is no evidence to date that qualitatively altered gene products are involved[1]. Much emphasis therefore rests on quantitative aspects of *c-onc* expression. Even this phenotypic distinction between leukaemic and normal cells could be small or perhaps only evident in the time frame, i.e. equivalent normal cells may express similar levels of *c-onc* gene products but only transiently.

D. Epilogue

Several of the ALL-associated membrane antigens have now been biochemically characterised and their control mapped to particular chromosomes (Table 2).

[1] An important example of such an alteration has however recently been reported [43]

Whether any of these proteins has any important regulatory role in differentiation or are even *c-onc* gene products is at present unknown. One of these structures does have a definite function. The monclonal antibody OKT9 identifies the transferrin receptor [14]; this observation has enabled rapid progress to be made in the biochemical studies of this receptor [28] and also facilitated the mapping of controlling (presumably structural) genes to chromosome 3 [29]. We have also suggested that the transferrin receptor may serve as a common 'target' structure on malignant and normal cells for so-called natural killer (NK) cells [30].

There are still many gaps in our understanding of lymphoid malignancy and of normal lymphopoiesis. Compared with myelopoiesis for example (see paper by Metcalf in this volume) we have little insight into soluble regulators of early lymphocyte development. Despite these limitations lymphoid malignancy in humans provides, I believe, an excellent example of a disease whose molecular, cellular and clinical complexity can be best understood in relationship to normal cellular differentiation.

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Bone Marrow Transplantation in Leukemia

E. D. Thomas

A. Introduction

Studies in the mid-1950s using rodent models led to recognition that living bone marrow cells could be transplanted from one animal to another (reviewed in [18]). The clinical applications for replacement of marrow damaged by disease or its therapy were immediately apparent. However, more than a decade of research was required before the principles of transplantation biology, human tissue typing, and the supportive care of the patient without marrow function were sufficiently established for marrow grafting for therapeutic purposes to become a realistic clinical modality.

The underlying concept of marrow transplantation is to destroy malignant cells without regard for marrow toxicity with restoration of marrow function by transplantation of normal marrow cells. The goal is to achieve a maximum anticancer effect without the limitations imposed by the marrow toxicity which characterizes most therapeutic agents.

B. Allogeneic Marrow Transplants

I. Rationale for Chemoradiotherapy

In early studies of patients with acute leukemia in end-stage relapse, it was considered necessary to administer total body irradiation (TBI) as quickly as possible in the hope that a marrow graft would be functional before the patient died of problems related to marrow failure. Accordingly, 1000-rad TBI was administered over a period of approximately 4 h [19]. The TBI caused the sudden destruction of large numbers of leukemic cells, resulting in acute toxic reactions. To spread the destruction of leukemic cells over a longer time period and to kill more leukemic cells, the Seattle team initiated the use of a large dose of cyclophosphamide before the TBI [20]. The basic regimen consisted of cyclophosphamide 60 mg/kg body wt. on each of 2 days followed 3 days later by the administration of 1000-rad TBI. There is now an extensive experience with this regimen in a number of marrow transplant centers. The greatest experience has been with an allogeneic marrow graft from an HLA-identical sibling given within 24 h after the TBI. The clinical experience in Seattle with this basic regimen, in some patients combined with additional chemotherapeutic agents, is summarized.

II. Transplantation for Acute Leukemia in End-Stage Relapse

Fifty-four patients with acute nonlymphoblastic leukemia (ANL) and 46 patients with acute lymphoblastic leukemia (ALL) were given cyclophosphamide 60 mg/kg×2, 1000 rad TBI, and marrow from HLA-identical siblings [20]. There were many early deaths from advanced illness and subsequent deaths from graft-versus-host disease (GVHD), opportunistic infection, and recurrence of leukemia. However, six patients with ANL and seven patients with ALL are alive in unmaintained remission 6–10 years later. Although the fraction of long-term survivors is low, these patients are unique in that no other form of therapy has resulted in prolonged unmain-

tained disease-free survival in relapsed patients. Actuarial analysis demonstrates a flat long-term disease-free plateau and provides evidence that these patients are cured of the disease.

III. Transplantation for ANL in First Remission

Since some patients in the end stage of the disease could be cured by combined chemoradiotherapy and allogeneic marrow transplantation, we initiated studies of marrow grafting in patients with ANL in first remission [21]. When these studies were begun, there were almost no reports describing median remission durations longer than 1 year. It seemed ethically acceptable therefore to carry out these studies in these patients. The first group of 19 patients was reported 3 years ago, and three additional patients were transplanted while that report was in press. Twelve of these 22 (55%) are alive in unmaintained remission 4–6 years after transplantation. Only one patient has significant chronic GVHD with a Karnofsky score of 80%.

IV. Transplantation for ALL in Second or Subsequent Remission

Patients with ALL who relapse have a grim prognosis. Subsequent remissions can frequently be induced but tend to be short in duration. We initiated a study for patients with ALL in second or subsequent remission in order to carry out the marrow graft when the patient was in good condition and when the possibility of cure might be increased because of the minimal burden of leukemic cells in the body [22]. Of the first 22 patients, the median remission duration after grafting was 1 year, and six patients became long-term survivors. The apparent cure rate of 27% is a significant achievement, but we were disappointed by the fact that leukemia recurred in eight of these patients. A Kaplan-Meier analysis indicated that 60% of these patients would suffer a relapse of leukemia if other causes of death were eliminated. In a subsequent study it was shown that marrow transplantation in remission was superior to chemotherapy for patients with ALL who have relapsed at least once [11].

V. Transplantation for Chronic Myelogenous Leukemia (CML) in Blast Crisis

Our initial efforts to carry out marrow transplantation in patients in blast crisis after failure of chemotherapy were unsuccessful [6]. Of 12 patients, only one had a remission beyond 1 year, and he died at 16 months of recurrent leukemia. In the more recent series of patients in which the marrow graft was undertaken before combination chemotherapy had been administered, the results have improved ([7] and unpublished). These studies involved the usual two doses of cyclophosphamide followed by fractionated irradiation, either 1200 rad or 1575 rad. Eight of 22 patients are alive in remission from 4 to 48 months after grafting.

VI. Transplantation for CML in Chronic Phase

CML in chronic phase is not actually a "chronic" disease. The median survival time is 2 or 3 years, and there are no cures by conventional therapy. We began studies of this disease in a series of 12 patients who had cytogenetically normal identical twins to serve as marrow donors [8]. Dimethyl myeleran, 5 mg/kg, was administered before the regimen of cyclophosphamide and 1000-rad TBI. One patient died of an interstitial pneumonia, one died of cytogenetic relapse and subsequent blast crisis, and two are living and well but have had recurrence of the Philadelphia chromosome. Eight patients are living, well, and cytogenetically normal 24–68 months after transplantation.

Encouraged by an apparent ability to eradicate the abnormal clone of leukemic cells in most patients, we began a study of marrow grafting for patients with CML in chronic phase with HLA-identical siblings as donors [7]. The first three patients were prepared with cyclophosphamide followed by 1000-rad TBI, and one patient is living and well 35 months later. Two patients died early, one of interstitial pneumonia and one of GVHD.

The current study for CML patients in chronic phase consists of the two doses of cyclophosphamide followed by 200-rad irradiation on each of 6 days; patients are

then randomized to receive methotrexate or cyclosporine for prevention of GVHD. Thirteen patients have been entered on the study. Four died of interstitial pneumonia, and nine are living with a graft and without the Philadelphia chromosome 5–20 months after grafting. A preliminary report from the Toronto marrow transplant team describes 11 patients with CML in the accelerated phase [12]. The preparative regimen usually included cytosine arabinoside (100 mg/m^2 per day×5), cyclophosphamide (60 mg/kg per day×2), and 500-rad TBI. Seven patients were alive without the Philadelphia chromosome 2–26 months after grafting. Another preliminary report from the UCLA marrow transplant team described five patients with CML in chronic or accelerated phase prepared with the two doses of cyclophosphamide and 1000-rad TBI and given HLA-identical sibling marrow [4]. All five were alive and without the Philadelphia chromosome 6–15 months posttransplant. Although a longer follow- up period will be necessary, it appears that more than half of the patients with CML can be cured of the disease but that some patients will die early of complications of the transplant procedure.

VII. Recurrence of Leukemia

The recurrence of leukemia after marrow transplantation for patients with ANL in first remission is a relatively minor problem since only 10% of these patients are destined to have a recurrence as determined by an actuarial analysis. The long-term survival and apparent cure rate is 50%–60%. For all other types of leukemia, when relapse has occurred at least once, whether the patient is transplanted in remission or in relapse, recurrence of leukemia has been observed in approximately 60% of the patients. The long-term disease-free survival and apparent cure rate is approximately 10%–30% [1, 3, 9].

Seven cases of recurrence of leukemia in the donor-type cells have been reported (reviewed in [16]). Two of these recurrences were an immunoblastic lymphosarcoma type, one associated with Epstein-Barr viruses. The other occurrences have been of the original leukemic type, including both ALL and ANL. In a study of recurrent leukemia in patients with a donor of opposite sex, the Seattle group has recognized three recurrences in donor cells among 54 such transplants. Thus, it appears that approximately 5% of the recurrences may be expected to be in the donor-type cells. The mechanism of these recurrences in donor-type cells is, of course, unknown. Present speculations suggest that some type of transfection may be involved.

VIII. Acute GVHD

Acute GVHD involves the skin, the liver, and the gut as target organs and is associated with severe immunodeficiency [18]. Approximately 60% of the patients receiving a marrow transplant from an HLA-identical sibling and treated postgrafting with methotrexate will show no evidence of GVHD or only grade I GVHD. Forty percent will have more severe GVHD with multiple organ involvement. Treatment of acute GVHD has been attempted with prednisone, antithymocyte globulin, cyclosporine, cyclophosphamide, and various monoclonal antibodies. The response to treatment is variable and unpredictable.

IX. Chronic GVHD

About one-third of the patients who live beyond 100 days postgrafting will display some evidence of chronic GVHD. Chronic GVHD typically presents a scleroderma-like involvement of the skin associated with sicca. Chronic GVHD may also involve the liver or the gut. About 80% of the patients with chronic GVHD will respond to therapy with azathioprine and prednisone or cyclophosphamide and prednisone [17].

X. Opportunistic Infections

Patients with a marrow graft from an HLA-identical sibling are profoundly immunodeficient in the first 100 days after grafting, and 1 year is required for full recovery of immunologic function [14]. The presence of GVHD, either acute or chronic, is associated with further suppression of immune function. During the period of immunodeficiency, patients are susceptible to infection with a broad range of bacterial, viral, and fungal infections [13].

XI. Graft Versus Leukemia

It has long been known from studies in rodents that an allogeneic graft may have an antileukemic effect [2]. With better survival of patients with GVHD, it has now been possible to show that the presence of GVHD indicates a lower incidence of recurrence of leukemia after grafting [23].

XII. Cyclosporine

Cyclosporine is a fungus-derived antibiotic with profound immunosuppressive properties without marrow toxicity. Preliminary and uncontrolled trials of this agent indicate that it is of value in preventing GVHD and in treating established GVHD [15]. A prospective trial has been underway in Seattle for past 1½ years. Patients are randomized to treatment with cyclosporine after grafting in comparison to the standard postgrafting methotrexate regimen. With some 60 patients entered into the study, the survival curve of the two groups is not statistically significantly different.

XIII. Monoclonal Antibodies

Many monoclonal antibodies which react with various epitopes on the surface of T cells are now available. Since GVHD is presumed to be mediated by T cells, it is reasonable to attempt to prevent GVHD by in vitro treatment of the donor marrow with monoclonal anti-T cell antibodies as well as the in vivo administration of these antibodies for the treatment of established GVHD. Although the use of monoclonal antibodies is being studied in many marrow transplant centers, definitive reports have not yet appeared.

XIV. Haploidentical Marrow Donors

The Seattle Marrow Transplant Team began 5 years ago a cautious exploration of family-member donors with one HLA haplotype genetically identical with the patient and the other HLA haplotype phenotypically identical at two of the three major HLA loci [5]. Some 80 patients with leukemia have now been transplanted from donors of this type and, overall, the results are much more a reflection of the type and stage of the disease than of the transplant donor.

XV. Unrelated Donors

Three years ago the Seattle Transplant Team carried out a transplant for a patient with ALL using a totally unrelated donor [10]. The transplant was successful, and the recipient had no GVHD. This case illustrated the feasibility of using as a donor a completely unrelated individual.

C. Summary

Marrow grafting is now an established treatment for patients under the age of 50 with acute leukemia and a suitable marrow donor. For all patients who have relapsed at least once, marrow grafting offers the possibility of cure of approximately 20%–30% of these patients, which cannot be achieved by any other regimen yet reported. Although still somewhat controversial, it appears that marrow grafting is also the treatment of choice for younger patients with ANL in first remission since approximately 50%–60% of these patients can be cured. The problems associated with marrow grafting are largely those of failure to eradicate the malignant disease and of transplantation immunobiology. Progress is being made on solving these problems, and the ever-increasing number of marrow transplant centers involved in the study of these problems promises rapid progress in this field.

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Nucleotide Metabolism and Enzyme Inhibitors in Thymic Acute Lymphoblastic Leukaemia *

A. V. Hoffbrand, D. D. F. Ma, and H. G. Prentice

A. Introduction

It is now clear that not only the immunological but also the biochemical characteristics of leukaemia cells are similar to those of the normal cells from which they arise. This has been most clearly established for the lymphoid malignancies and particularly in thymic-derived acute lymphoblastic leukaemia (Thy-ALL). Close similarities between Thy-ALL leukaemic blast cells and early cortical thymocytes in surface membrane antigen phenotype, in terminal deoxyribonucleotidyl transferase (TdT) content and in pattern of purine enzymes have recently been established [5]. These findings have helped to establish the exact cell of origin of Thy-ALL and made possible its diagnosis by single cell analysis in bone marrow or extra medullary sites. They have also led to the treatment of this disease with 2′deoxycoformycin (dCF), a specific inhibitor of the purine degradative enzyme, adenosine deaminase (ADA). Part of the stimulus to this research into the biochemistry of the thymus and the treatment of Thy-ALL with dCF has arisen from observations on children with severe defects of immune development due to congenital deficiency of ADA or of a second purine degradative enzyme, purine nucleoside phosphorylase (PNP). Lack of these enzymes causes absence of T- and B-lymphocytes or of T-lymphocytes respectively but other functions of the body, including haemopoiesis are largely unaffected.

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B. Purine Enzyme Patterns: Normal Tissues and Established Cell Lines

Studies in rats [1] and more recently in humans [12] have shown that the activities of several enzymes involved in purine metabolism differ widely between different lymphocyte subpopulations, both between B and T cells and in the B- and T-cell lineages, according to the degree of differentiation of the cell studied. ADA is concerned with the degradation of deoxyadenosine and adenosine to deoxyinosine and inosine respectively. It is present in all tissues, but its activity is highest in cortical thymocytes and decreases as T cells mature. In humans, the earliest cortical thymocytes (early cortical blasts or "prothymocytes") have the highest level of all, whereas in rats, immature cortical thymocytes and bone marrow prothymocytes have lower levels than the majority of thymic cortical cells. ADA activity in both humans and rats is higher in mature T cells than in B cells.

PNP is a consecutive enzyme with ADA in purine degradation, breaking down deoxyinosine and inosine to hypoxanthine, and also deoxyguanosine, guanosine and xanthosine to xanthine. In rat lymphocyte populations, a reciprocal relationship exists between ADA and PNP, since cortical thymocytes have high ADA and low PNP levels whereas medullary thymocytes and circulating T cells have high PNP and low ADA levels. Human thymocytes show a similar reciprocal relationship except in prothymocytes which have high PNP as well as high ADA levels.

A third enzyme, 5′-nucleotidase, exists on the surface of lymphocytes which is capable of degrading deoxynucleoside monophosphates to the corresponding deoxynucleosides. The exact biological function of this ectoenzyme is unclear. The activity amongst T-cell subpopulations in humans closely parallel that of PNP, being low in cortical thymocytes and higher in mature T cells [12]. 5′NT activity is, however, substantially greater on the surface of B- than T-lymphocytes and among mature T-lymphocytes; the activity is greater on T suppressor ($OKT8^+$) cells than on T helper ($OKT4^+$) cells [15]. Although 5′NT has been found low in the lymphocytes of patients with congenital agammaglobulinaemia, this is thought to be more a result of the lack of B cells than a cause of the condition. The activity of a recently described endonucleotidase [2] among different lymphoid populations is as yet unknown.

Other enzymes concerned in deoxynucleotide degradation are also more active in B cells than T cells. These include ecto-ATPase and thymidine phosphorylase. Moreover, studies with established cell lines have shown that immature T cells are unable to functionally compartmentalise thymine nucleotides into a degradative as well as a synthetic compartment, whereas other cell types have a degradative compartment for deoxynucleotides as well as a synthetic compartment destined to be incorporated in DNA [22]. Recent studies with established cell lines also show that as cells mature in B-cell development from c-ALL through pre-B-ALL to mature B cells, so the ability to degrade DNA precursors increases [23]. PHA-transformed T-lymphocytes, normal human bone marrow cells and myeloid cell lines all also have substantial degradative compartments. Virtually all the thymine nucleotides in Thy-ALL lines, and presumably in early thymocytes (although this has not been studied directly) are destined to be incorporated into DNA, however. This is thought to be due to the operation of a highly efficient multi-enzyme complex in Thy-ALL cells, synthesising thymine nucleotides and providing dTTP at the DNA replication fork without leakage of distal precursors to a degradative compartment. Such efficient complexes are likely to operate in supplying the other three deoxynucleoside triphosphates (dNTP), deoxyadenosine- (dA-), deoxyguanosine- (dG-) and deoxycytidine- (dC-) TP. Thus, Thy-ALL and normal thymic cortical cells have low levels of deoxyribonucleoside- and deoxyribonucleotide-degrading enzymes except for ADA. They also lack a degradative compartment for syphoning off excess deoxyribonucleotides. They are, therefore, peculiarly prone to dATP or dGTP toxicity if ADA of PNP are absent or inhibited, when excess amounts of dATP or dGTP respectively are built up.

C. Terminal Deoxynucleotidyl Transferase

This unusual DNA polymerase, like ADA, is present in high concentrations in prothymocytes and cortical thymocytes. It is absent from mature T cells. The only other normal cells containing TdT are a small proportion of bone marrow cells and these have been shown to exhibit the phenotype of c-ALL or pre-B-ALL [7], which have both been identified as early B cells because of the gene rearrangements they show. Thus TdT is a marker of early cells in the B- or T-cell lineages. The normal function of TdT is unknown, although it has been suggested to play a role in generation of immune diversity by altering the base composition of DNA. The optimum substrate for TdT is dGTP and Ma et al. [13] have recently speculated that in early thymocytes, TdT may polymerise excess dGTP or other dNTP to make single-stranded DNA polymers which if not incorporated into double-stranded DNA may be subsequently degraded with release of potentially toxic intracellular concentrations of the corresponding deoxynucleosides and deoxynucleotides.

D. ADA and PNP Deficiencies: Mechanisms of Cell Death

Children born with ADA deficiency show lack of T- and B-cell development. The lack of B cells may be due to absence of both suppressor and helper T cells needed

for B-cell maturation. The main mechanism by which ADA deficiency is toxic is thought to be dATP accumulation, due to failure of degradation of deoxyadenosine, with consequent allosteric inhibition of ribonucleotide reductase and failure of supply of the other three deoxynucleoside triphosphates with consequent cessation of DNA replication. Additional toxicity may be due to inhibition of *S*-adenosyl methionine (SAM) mediated methylation reactions because of inhibition of *S*-adenosyl homocysteine hydrolase by excess deoxyadenosine. Lowered ATP and raised cyclic AMP levels have also been found in ADA-deficient tissues and these may inhibit a wide variety of reactions in both replicating and non-replicating cells (see [6] for review). Most recently, Fox et al. [3] have shown that the combination of deoxyadenosine and ADA inhibition by erythro-9-(3-(2-hydroxynosyl)) adenosine leads to arrest of Thy-ALL lines in vitro in the G_1 phase of the cell cycle. This was associated with a rise in dATP in the G_1 phase, and this and the cell arrest could be prevented by deoxycytidine. Non-dividing T cells are also killed [8]. They have subsequently postulated that this may be due to incorporation of accumulated deoxyadenosine into the poly (A) tail of RNA with interference in the processing, transfer and transcription of messenger RNA [10].

PNP deficiency causes a much more selective lack of T cells with relative sparing of B cells and their function. Toxicity is thought to be mainly due to dGTP accumulation (at a later stage of T-cell maturation than in ADA deficiency) with inhibition of ribonucleotide reductase.

E. Enzyme Patterns in Leukaemic Cells

The pattern of purine enzymes and TdT in leukaemic cells shows a remarkably close similarity to the normal counterparts of these cells. Thus, Thy-ALL blasts, like early cortical thymocytes have, in general, high levels of ADA and TdT but lower concentrations of PNP and 5′NT [20]. On the other hand, more mature T-cell tumours (e.g. T-cell CLL, Sezary cells and T-prolymphocytic leukaemia) are TdT negative, have only moderately high ADA activity and show higher PNP and 5′NT levels than in Thy-ALL [14]. Although the normal bone marrow precursor cells from which AML and c-ALL arise have not been isolated in sufficient numbers and purity for biochemical analysis, it seems probable that the purine enzyme pattern and TdT content of blast cells in AML and c-ALL reproduce those of early bone marrow myeloid and lymphoid progenitors respectively, AML typically being TdT negative with moderately raised ADA, PNP and 5′NT and c-ALL being TdT positive with ADA lower, PNP and 5′NT higher than in Thy-ALL.

Nucleoside incorporation studies in blast cells of Thy-ALL have shown a pattern distinct from the blast cells in other types of acute leukaemia. Incorporation of deoxycytidine is raised and of thymidine low, so that the ratio of uptake of deoxycytidine to thymidine is higher in Thy-ALL than in c-ALL or AML blasts [17]. The levels of all four deoxynucleoside triphosphates (dNTP) are also usually considerably higher in Thy-ALL than in other acute leukaemias. This may partly be due to the larger number of cells in cycle in Thy-ALL, but the particularly high levels of dNTP suggest that this may also be due to greater synthetic and less degradative capacity of Thy-ALL blasts for deoxyribonucleotides. Normal thymocytes also show high concentrations of the dNTP [16].

F. Deoxycoformycin Therapy

The known dependence of early thymocytes on ADA led to the development of a specific ADA inhibitor 2′deoxycoformycin (dCF) as a potential immunosuppressive agent and for the treatment of thymic-derived tumours. A number of groups in the United Kingdom and the United States have used dCF to treat Thy-ALL and found the drug to be effective in obtaining a remission in the majority of cases, even those resistant to other forms of chemotherapy. In our own experience, a remission was obtained in 7 of 12 patients using a 5-day course of the drug at 0.25 mg/ kg each day. Two cases proved resistant and in three a partial remission

was obtained with one or two courses [18]. Patients with other T-cell tumours, e.g. T-prolymphocytic leukaemia and mycosis fungoides have also responded to dCF whereas cases of c-ALL and AML have, in our hands, proved resistant. Others, however, have obtained responses in c-ALL [4] and even in B-cell CLL [9].

The mechanism of cell killing has been analysed by serial biochemical studies. Blast cell death more closely correlates with dATP rise than with *S*-adenosyl homocysteine hydrolase inhibition [19]. Indeed, a predictive test for response based on the degree of dATP rise in blasts incubated with dCF and deoxyadenosine in vitro has been devised [18, 21].

G. Side Effects

The effects of dCF therapy in Thy-ALL on tissues other than the leukaemic cells could not have been predicted from observations on ADA-deficient children. Iritis, hepatic abnormalities, haemolysis (in 9 of 17 patients studied by Prentice et al. [18]), central nervous system toxicities and renal abnormalities including renal tubular necrosis have all been described. The mechanisms for these toxicities are not clear. Reduced red cell ATP concentrations have been demonstrated and postulated to lead to haemolysis. Interference with cyclic AMP and SAM-mediated reactions are further possibilities. Hyperuricaemia was a problem in early studies, but since the use of allopurinol this has been prevented.

H. In Vitro Removal of Thy-ALL Blasts

The selectivity of dCF therapy for Thy-ALL with sparing of haemopoiesis has aroused interest in the possibility of using dCF in vitro to remove selectively residual Thy-ALL blasts from bone marrow prior to autologous bone marrow transplantation. However, studies in cell lines have shown that ADA inhibition alone in vitro does not lead to death of T cells or other cell types. On the other hand, deoxyadenosine is toxic to cells in vitro and T cells are susceptible at lower concentrations than B cells or other cell types. Studies of the combination of dCF ($10^{-5}M$) and deoxyadenosine ($10^{-4}M$) have shown toxicity to Thy-ALL lines in vitro with considerable selectivity, growth of c-ALL, B- and myeloid cell lines being far less inhibited [11].

The use of the combination of dCF and deoxyadenosine in vitro for selective killing of residual Thy-ALL blasts prior to autologous bone marrow transplantation has not yet been used because of the long incubation period necessary to achieve substantial cell killing. For established Thy-ALL cell lines, 72 h incubation at 37 °C is needed to achieve over 80% cell death and studies of blast cells from individual patients with Thy-ALL have shown a similarly long incubation period to be necessary (Ma, Sylwestrowicz and Hoffbrand, unpublished observations).

It is not considered practical to maintain bone marrow in culture at 37 °C in vitro for 3 days free from contamination and with sufficient preservation of normal haemopoietic stem cells to ensure successful engraftment.

I. Conclusion

Many of the biochemical features of Thy-ALL reproduce those of early cortical thymocytes, and result in Thy-ALL blast cells being exquisitely dependent on adenosine deaminase to degrade deoxyadenosine. These observations have led to the use of deoxycoformycin, a specific ADA inhibitor, in treatment of Thy-ALL. Further studies of the biochemical make-up of the blast cells in different types of leukaemia, particularly of the organisation of DNA and RNA synthesis and degradation, are needed in order to improve the design of chemotherapy with antimetabolite and other drugs in these diseases.

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Selective Killing of Leukemia Cells by Inhibition of TdT *

R. McCaffrey, R. Bell, A. Lillquist, G. Wright, E. Baril, and J. Minowada

A. Introduction

Terminal deoxynucleotidyl transferase (TdT) is a unique DNA synthetic enzyme whose expression in normal cells is restricted to subsets of primitive lymphocytes, and among neoplastic cells is found only in the blast cells of some forms of acute leukemia and diffuse lymphoma [1–5]. This polymerase has achieved increasing attention over the last several years from both basic biologists and physicians caring for patients with malignant hematologic disease. For biologists it has emerged as a useful biochemical marker for subsets of pre-B and pre-T cells, and has played an important role in studies aimed at dissecting the ontogeny of the lymphoid system [6, 7]. In clinical medicine, blast cell TdT assays have been shown to be useful in the subclassification of acute leukemias and diffuse lymphomas, and TdT status has been used to assign patients to therapeutically meaningful categories [8, 9].

Although the existence of this enzyme has been known for over 20 years, the function it subserves in the cells in which it is found is presently unknown. Its strict limitation in normal animals to lymphoid cells during the early phases of their differentiation suggests that it may play a critical role in this process. Presumably TdT subserves a similar role in leukemia cells, although the process of differentiation is itself disturbed. Whatever its role might be, we asked whether inhibition of TdT in leukemic cells might constitute a lethal event to such cells. We therefore began a study to find a specific TdT inhibitor. With such a probe one would be able to examine a variety of cellular processes and functions in TdT-positive cells, and gain insight into the role of this enzyme in both normal and malignant cells.

B. In Vitro Inhibition of TdT Catalysis

We began our search for TdT inhibitors by screening a series of 6-anilinouracil derivates for their ability to inhibit TdT catalysis as measured in a standard biochemical reaction. This series of compounds was selected for our initial screen because of the work of Wright, Baril, and Brown [10] with one such compound, 6-butylanilinouracil, which they showed to be a specific inhibitor of HeLa cell DNA polymerase α. The other constitutive HeLa cell polymerases, polymerases β and γ, were not inhibited. Furthermore, this compound, in a range of concentrations which were inhibitory to in vitro enzyme catalysis, also strongly inhibited cell proliferation in nonsynchronous HeLa cell cultures in a dose-dependent fashion. Similar data on growth inhibition were also generated with KB cells lines. We thus proceeded to test related analogues for their effect on TdT.

Fifteen uracil analogues were studied. Each was employed at 200 μmolar final concentration in 1% DMSO (required for solubility) in our standard TdT biochemical assay [1]. Control TdT reactions were also run in the presence of 1% DMSO. Two

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Designator	Name	nmoles ^{3}H-dGMP inc.
Control		1.22
GW-9E	6-anilinouracil	1.21
GW-7B	6-(benzylamino)uracil	1.03
GW-7C	6-(phenetylamino)uracil	1.31
GW-11D	6-(p-butylanilino)uracil	1.12
GW-22E	6-(p-hydroxyanilino)uracil	1.21
GW-16C	6-(p-acetamidobenzylamino)uracil	1.43
GW-18B	6-(cyclohexylamino)uracil	1.31
GW-20B	6-(cyclohexylmethylamino)uracil	1.33
GW-18E	6-(*n*-pentylamino)uracil	1.20
GW-22A	6-(iso-pentylamino)uracil	1.21
GW-17B	6-(3′,4′-trimethyleneanilino)uracil	1.23
GW-28A	6-(*d*-naphthylamino)uracil	1.24
GW-33E	5-(p-methoxybenzyl)-6-aminouracil	1.20
GW-17E	6-(p-methoxyanilino)uracil	0.51
GW-18C	6-(p-aminoanilino)uracil	0.69

Table 1. Effect of uracil analogues on TdT activity

compounds, designated GW-17E and GW-18C, showed significant inhibition in this screen (Table 1).

This inhibition by GW-17E and GW-18C was specific for TdT. Data for representative experiments involving polymerases α, β, and γ are shown in Table 2. Both compounds inhibited TdT in a dose-dependent manner (Fig. 1). Avian myeloblastosis virus reverse transcriptase was not inhibited by either GW-17E or GW-18C.

The general structure of compounds GW-17E and GW-18C are shown Fig. 2. For compound 17E the R denotes a methoxy substitution; for 18C the R denotes an amino substitution. Para position substitutions are critical for inhibitory activity. Moving the methoxy or amino group to another position on the aniline ring results in loss of inhibitory activity.

In these preliminary studies inhibition was neither initiator dependent nor substrate dependent: it occurred with both oligo(dA) and single-stranded DNA as initiators and with dGTP and TTP as substrates. The TdT used in these experiments was purified (using ion-exchange and affinity chromatography [12]) from both calf

Table 2. Specificity of inhibition of TdT by GW-17E and GW-18C

Enzyme	CPM ^{3}H-dNMP incorporated		
	Control	+17E	+18C
TdT	12,130	4,900	3,100
Pol α	15,040	15,217	15,417
Pol β	5,221	5,492	5,218
Pol γ	7,490	7,223	7,165

Effect of 400 μmolar inhibitor on activity of homogeneously purified human leukemic TdT and HeLa cell DNA polymerases. TdT, pol α, pol β, and pol γ were assayed as previously described [10, 11]

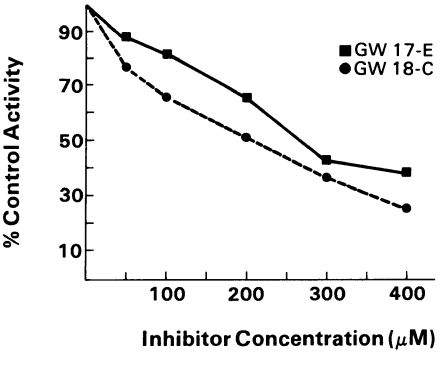

Fig. 1. Inhibition of TdT by 6-(p-methoxyanilino)uracil (GW-17E) and 6-(p-aminoanilino)uracil (GW-18C)

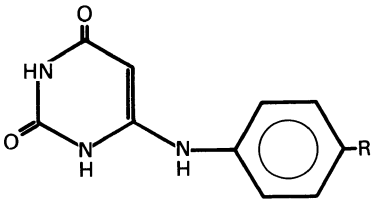

Fig. 2. General structure of compounds GW-17E and GW-18C. For 17E the R denotes a methoxy substitution, and for 18C the R denotes an amino substitution

thymus gland and human leukemia cells. Formal inhibition studies to determine the nature of the inhibition (competitive versus noncompetitive K_i values) are in progress.

C. Effect of GW-17E and GW-18C on Cell Proliferation

We next attempted to extend these observations to additional TdT-positive and TdT-negative cell lines. We studied 30 cell lines initiated from patients with various forms of acute leukemia and maintained at Roswell Park Memorial Institute. Twenty-one lines were TdT positive, nine were TdT negative. However, the requirement for 1% DMSO for the solubility of GW-17E and GW-18C turned out to be toxic for these cell lines: control cultures grew with extreme variability in the presence of 1% DMSO, making the interpretation of inhibitor effects impossible.

Table 3. Effect of GW-17E and GW-18C on cell proliferation

Cell line	Source	TdT status	Growth
HeLa	Human	Negative	Unchanged
L1210	Murine	Negative	Unchanged
LE-4	Murine	Positive	Markedly inhibited
HPB-ALL[a]	Human	Positive	Control cultures fail to grow in 1% DMSO
JM[a]	Human	Positive	

[a] The data noted for the two human leukemia cell lines are representative of all 30 lines studied

Work in progress now is directed at the development of compounds related to GW-17E and GW-18C, which are both inhibitory and water soluble. One such compound, designated 20-DN (Fig. 3), has been identified. At 100 μmolar concentration this compound inhibits TdT in vitro by 85% of control, while polymerases α, β, and γ are not inhibited, even at 500 μmolar concentration.

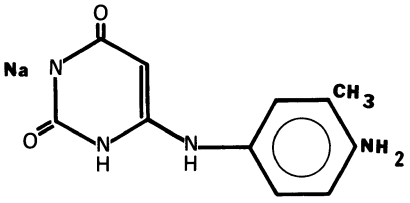

Fig. 3. Structure of compound GW-20DN

It should be possible to determine, using a panel of human and animal TdT-positive and TdT-negative cell lines, whether the results we have seen with HeLa, L1210, and EL-4 cells are general and related to TdT states, and not restricted to the lines studied to date.

D. Conclusion

The physiologic function of TdT in cells in which it is expressed, either leukemic or normal, is presently unknown. Its strict limitation in normal animals to lymphoid cells during the early phases of their differentiation suggests that it may play a critical role in this process. Presumably TdT subserves a similar role in leukemia cells, although the process of differentiation in these cells is itself disturbed. Whatever its role might be, we have asked whether inhibition of TdT in leukemic cells might constitute a lethal event to such cells. Our preliminary data on growth inhibition by GW-17E and GW-18C of a murine TdT-positive cell line suggests that this may indeed be the case.

The development of potent and specific TdT inhibitors will provide a critical tool in the dissection of the biological role of TdT. Such compounds may be of therapeutic use

in TdT-positive malignant states as definitive or adjunctive therapy, or possibly in the in vitro destruction of TdT-positive malignant cells in bone marrow prior to autologous grafting. Although TdT-positive normal cells, in addition to TdT-positive malignant cells, might be eliminated by therapy of this short, the fact that pluripotent stem cells are TdT negative suggests that the normal TdT-positive cell compartment would therefore be renewable.

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Poly(A)-polymerase Levels in Leukemia

M. Papamichail, T. Trangas, N. Courtis, C. Ioannides, H. Cosmidou, G. A. Pangalis, and C. M. Tsiapalis

A. Introduction

Formation of individual functional mRNA sequences in eukaryotic cells requires many steps in addition to transcription. These include RNA splicing, base modification, polyadenylation-de-adenylation, transport from nucleus to cytoplasm, and assembly into the polyribosomes. A number of recent reports indicate that various control mechanisms may operate in these several steps of mRNA maturation before its translation [1]. Elucidation of such control mechanisms concerning eukaryotic gene expression in addition to its biological interest may be clinically useful in lymphoid malignancies.

Polyadenylation of the 3′-hydroxyl end of HnRNA and mRNA could theoretically be regulated by poly(A)-polymerase [2, 3]. To clarify the possible involvement of this enzyme in mRNA maturation and stabilization we have carried out measurements of poly(A)-polymerase in various human leukemias. We found that acute leukemias have higher enzyme levels than those observed in chronic lymphocytic leukemias.

B. Results

As can be seen in Table 1 peripheral blood lymphocyte soluble cell extracts from patients with acute leukemia have higher poly(A)-polymerase activity than that observed in lymphocytes from chronic lymphocytic leukemia patients. The mean polymerase unit per milligram soluble protein in 30 cases of chronic lymphocytic leukemia was 7.03 whereas in acute leukemic cases it was 49.25. This difference of enzyme levels is statistically significant. Among the acute leukemias acute myeloblastic leukemia seems to have the highest enzyme levels. However, more cases must be studied to substantiate this conclusion. Peripheral blood lymphocytes from normal donors have very low enzyme activity. The polyadenylation reaction showed an absolute requirement for divalent cations (Mn^{2+} being better than Mg^{2+}) and exogenous initiator. There were no significant differences observed in the level of poly(A)-polymerase initiated with oligo (A) ($A_{\overline{10}}$) or poly(A)($A_{\overline{200}}$) with soluble extracts from acute and chronic leukemic patients. The oligo(A)-initiated polymerase activity from all soluble cell extracts shows linear incorporation of AMP for 1 h. In contrast the poly(A)-initiated polymerase activity

Table 1. Poly (A)-polymerase levels in various leukemias

Diagnosis	Enzyme units (nmol/h)/mg
Chronic lymphocytic leukemia [30][a]	7.03± 8.17[b]
Acute leukemias [14][c]	49.25±39.03[b]
Normal peripheral blood lymphocytes [7]	2.73± 2.67[b]

[a] Numbers in parentheses indicate the number of cases studied
[b] Level of significance 99% (mean±SEM)
[c] Acute leukemias include nine acute lymphoblastic leukemias (ALL), two acute myeloblastic leukemias (AML), and three chronic granulocytic leukemias (CGL) in blast crisis

from both acute and chronic leukemic soluble cell extracts shows linear incorporation of AMP for more than 1 h with an apparent initial lag phase. All enzyme assays may be carried out at protein concentrations of crude cell extract between 1 and 3 mg/ml. Preliminary data in our laboratory indicate differences in the mol.wt. of the enzyme between the acute and chronic cases. Also in acute leukemias the poly(A)-polymerase consists of two enzyme species whereas in the chronic leukemic cases only one of these can be detected (as reported elsewhere).

C. Conclusions

The results of this study indicate that peripheral blood lymphocytes of patients suffering from acute leukemia have higher poly(A)-polymerase activity than lymphocytes from CLL patients. Of the acute leukemias, the highest levels of the enzyme were observed in AML cases. However, more cases must be tested to prove this.

There is evidence that polyadenylation of HnRNA and mRNA is an early post-transcriptional process presumably mediated by poly(A)polymerase. It has also been suggested that poly(A) confers stability and consequently enhances translational efficiency of some mRNAs [4, 5, 6]. It could be assumed that high poly(A)polymerase levels result in increased poly(A) content of mRNA and HnRNA. Therefore, the observed high levels of poly(A)polymerase in rapidly proliferating acute leukemic cells, which have increased translational needs, possibly correlate with a longer lifetime of mRNA. The opposite occurs in CLL.

Elucidation of mRNA adenylation by poly(A)-polymerase and the subsequent functional lifetime of mRNA in various types of leukemia may shed light in understanding better the cellular basis of cell proliferation and have some clinical significance in lymphoid malignancies.

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Short-term Therapy for Acute Myelogenous Leukaemia in Younger Patients

T. A. Lister, A. Z. S. Rohatiner, M. L. Slevin, H. S. Dhaliwal, R. Bell, G. Henry, H. Thomas, and J. Amess

A. Introduction

It has now been repeatedly demonstrated that it is possible to achieve complete remission (CR) in the majority of younger adults with acute myelogenous leukaemia (AML) [4, 5, 7, 11, 12], and that approximately one-fifth of patients in whom this is achieved will continue well without recurrence for many years [2, 6, 8, 10]. These observations have naturally stimulated considerable research in to how these achievements may be translated into cure being probable for the majority rather than possible for the minority. In general, the trend has been to increase the quantity of initial therapy to the limits of bone marrow tolerance, which has been considerably extended by the availability of platelet concentrates and powerful broad spectrum antibiotics. In 1978 such an approach was introduced at St. Bartholomew's Hospital. In the light of there being no convincing evidence that maintenance therapy prolonged remission following very intensive initial treatment, and some (evidence) to the contrary [3, 9], it was decided to limit the duration of the programme to approximately 6 months by terminating all therapy after a maximum of six cycles of adriamycin (adria), cytosine arabinoside (araC) and 6-thioguanine (6-TG) given at approximately 3 weekly intervals.

Preliminary results were reported in April 1982 [1] and this report constitutes a follow-up of 98 patients under the age of sixty.

B. Materials and Methods

I. Patients

Ninety-eight consecutive patients aged 15–60 with newly diagnosed AML commenced treatment at St. Bartholomew's Hospital between April 1978 and November 1981 and form the basis of this report (Table 1).

Table 1. Pre-treatment clinical details; $n=98$ M : F 52 : 47

Blast count $\times 10^9$/litre		Platelet count $\times 10^9$/litre
Range	0 – 392	8 – 386
Mean	28	74
Median	5	42

F-A-B Classification

M	1	2	3	4	5	6	Total
	39	19	9	21	5	6	99

II. Treatment (Table 2)

Two combinations of adria, araC and 6-TG were employed sequentially between April 1978 and June 1980. The initial combination was the least intensive (B-IX) and modified in favour of the next (B-X) because inadequate aplasia was induced. This was abandoned because of unacceptable early mortality and B-IX re-instituted. The finding that the duration of remission was significantly longer in patients surviving to enter CR with B-X, and improvements in

Table 2. Details of treatment programmes

Drug	Dose (mg/m^2)	Days	Cycles
	B-IX		
Adriamycin	50	1	1+2
	50	1	3+4
	25	2	3+4
	50	1+2	5+6
Cytosine arabinoside	200	1–5	1–6
6-Thioguanine	200	1–5	1–6
	B-X		
Adriamycin	25	1, 2, 3	1–6
Cytosine arabinoside	200	1–7	1+2
		1–5	3–6
6-Thioguanine	200	1–7	1+2
		1–5	3–6
	B-Xb		

Doses of all three drugs the same as B-X; araC given by continous intravenous infusion; araC and 6-TG given for 7 days in all cycles

supportive care led to the introduction of an intensification of B-X to B-Xb.

The majority of patients treated between July 1980 and November 1981 received this modification of the B-X programme, whereby adria was given on the 1st and 2nd days (total dose 75 mg), araC was given by continuous intravenous infusion, and araC and 6-TG were continued for 7 days in all cycles. Four patients received adria on the first 2 days with araC at 100–150 mg/m^2 per day by continuous infusion for 10 days without 6-TG. AraC at a dose of 2 Gm/m^2 twice daily (given over 3 h) for 6 days was substituted for one consolidation course in three patients. For the purpose of this analysis, primarily concerned with determining whether there was an advantage in treating patients with chemotherapy including 7 days of araC as opposed to 5, all these patiens are considered together. A maximum of six cycles was prescribed over approximately 6 months, patients being observed subsequently without therapy prior to relapse. If an HLA identical donor was available, chemoradiotherapy followed by allogeneic bone marrow transplantation was performed (Dr. RL Powles) at the completion of chemotherapy. Such patients have been excluded from the analysis from the time of transplantation.

C. Results

Complete remission (CR) was achieved in 56/98 patients with the initial therapy and in a further three after the addition of high-dose araC to give an overall complete remission rate of 59/98 (60%) (Table 3). The reasons for failing to enter CR are shown in Table 4. No patient with a presenting blast cell count in excess of 100×10^9/liter entered CR.

Table 3. Frequency of complete remission

B-IX	B-X	B-XB	Total
30/37	11/27	18/34	59/98

B-IX vs B-X $P=0.01$; B-IX vs B-Xb $P=0.01$; B-X vs B-Xb n.s.

Table 4. Reasons for failure to achieve remission

	B-IX	B-X	B-Xb	Total
Resistant leukaemia	2	7	5	14
Fatal infection	1	3	6	10
Haemorrhagic death	3	3	1	7
Other	1	3	4	8
Withdrawn	–	–	3	3
Total	7	16	19	42

Table 5. Proportion of patients receiving total planned therapy

B-IX	B-X	B-XB
23/30	8/11	5/15

B-IX vs B-Xb $P=0.01$; B-X vs B-Xb $P=0.05$

Only 36/59 patients entering complete remission completed six cycles of therapy, the proportion being significantly lowest ($P<0.01$) for patients receiving the most intensive programme (B-Xb) (Table 5). Also the mean time to completion of three cycles of therapy was significantly longer for B-Xb (90 days) than B-IX (86 days, $P=0.01$) or B-X (75 days, $P=0.05$).

Twenty-one patients have continued in first unmaintained CR between 6 months and 4 years with a median follow-up of 18 months (Fig. 1). Thirty-eight have relapsed, one has died of septicaemia without re-

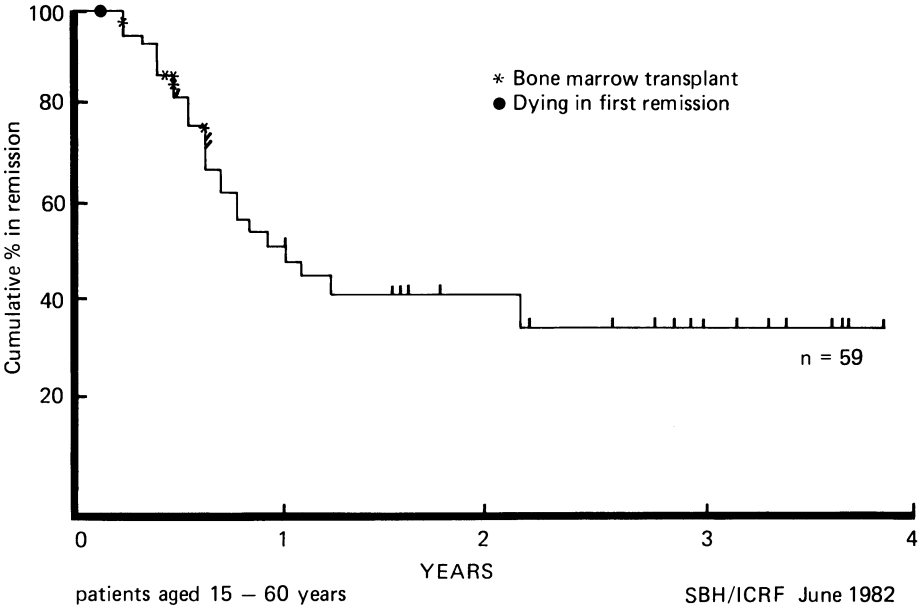

Fig. 1. Duration of first complete remission

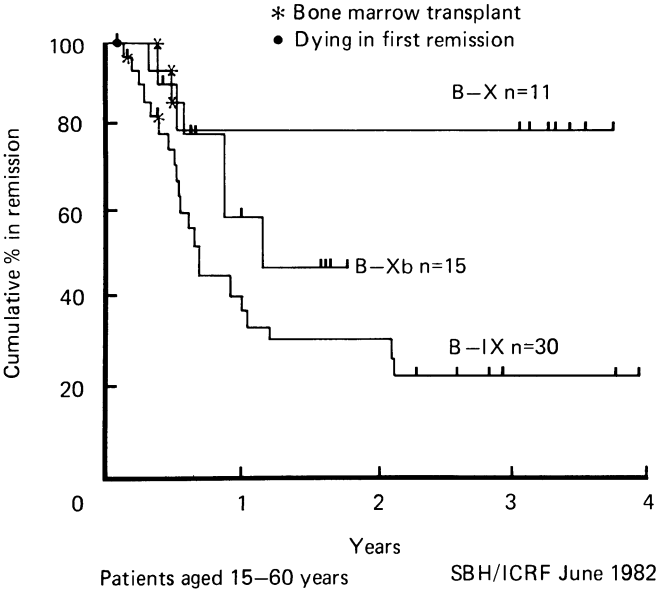

Fig. 2. Duration of first complete remission. Influence of treatment programme

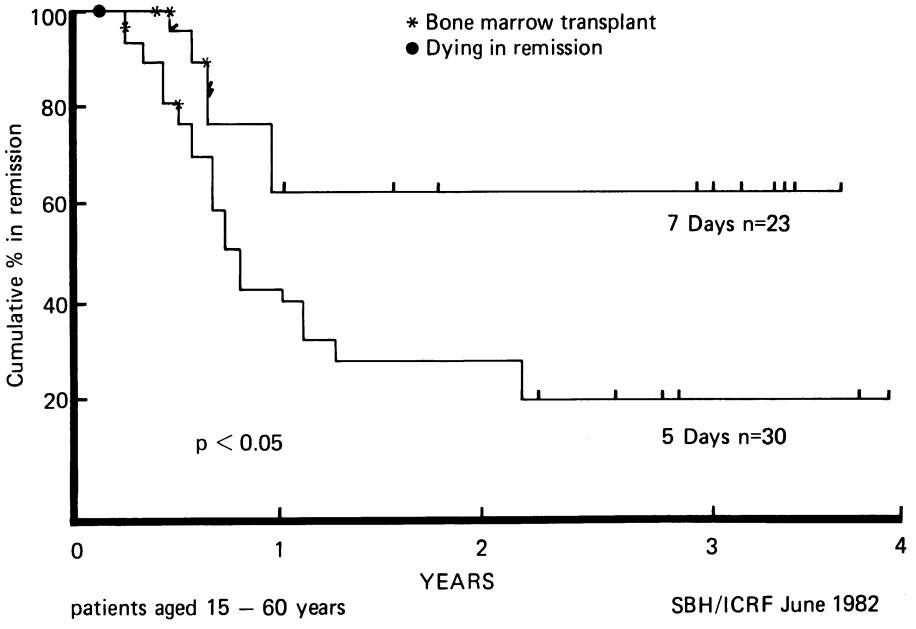

Fig. 3. Disease-free survival. Influence of initial therapy. araC: 7 days vs 5 days

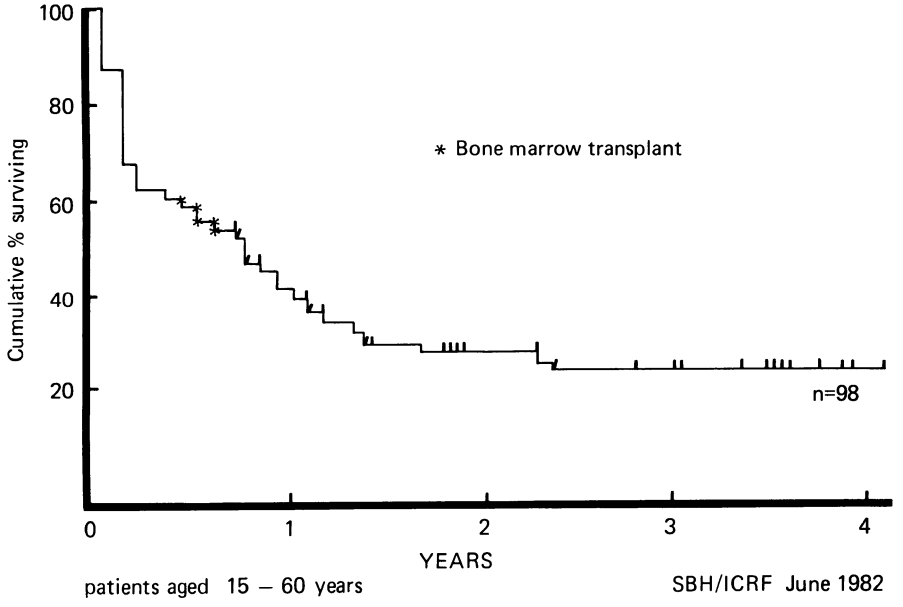

Fig. 4. Overall survival

lapse, and bone marrow transplantation was performed in five, all of whom have continued in remission but who have been excluded from the analysis from that time. The median duration of remission was 1 year. It was significantly longer for patients receiving B-X than those receiving either B-IX or B-Xb (not an entirely homogenous group) (Fig. 2). All three patients receiving B-Xb who proceeded to high-dose araC prior to entering complete remission have relapsed at 4, 8½ and 9 months. Compari-

son of the duration of remission of patients entering complete remission with a treatment programme contining 5 days araC and 50 mg/m² of adria in the first two cycles with those receiving one containing 7 days araC with 75 mg/m² of adria in divided doses reveals a significant advantage for the latter group ($P < 0.05$, Fig. 3)

The overall disease-free survival curve for all 98 patients commencing treatment in all of the studies in shown in Fig. 4.

D. Discussion

The complete remission rate for adults under 60 years of age treated at St. Bartholomew's Hospital has risen modestly from 43% (1974–1978) to 60% in the period 1978–1982, being highest in the group receiving the least intensive programme. Reducing the duration of therapy to approximately 6 months has not been associated with a reduction in the median duration of remission, nor in the proportion without relapse at 3 years. On the contrary, the current analysis shows a statistical advantage for patients receiving short-term therapy over those treated in previous studies at St. Bartholomew's Hospital. This is, however, the group treated most recently and obviously late relapses may occur. It is unlikely, however, that the results will be worse than those achieved previously, at least justifying the further investigation of short-term therapy. Comparison of the duration of remission attained with B-IX, X, and Xb shows an advantage for B-X over both the others in spite of the fact that B-Xb was nominally more intensive than B-X. There are several possible explanations for this. It may be that the very long remission duration of patients receiving B-X was a statistical fluke, in part a reflection of the small number of patients. Two other alternatives are possible. First, the premature termination of B-Xb prevented many patients receiving adequate therapy. Second, resistant leukaemia was allowed to develop by the prolonged intercycle time.

The fact that an advantage may be demonstrated for the 7 (or more) -day schedules, but not for those in which araC was given by continuous infusion, may be variously interpreted. It seems most likely that this paradoxical result is a reflection of the fact that 200 mg/m² araC when given by twice daily bolus injection for 7 days in combination with adria and 6-TG is just tolerable for six cycles at 3–4 weekly intervals, but intolerable when given by infusion. It may therefore be inferred that if the schedule of administration of choice is a 7-day continuous infusion, the daily dose of araC must be reduced, or the other drugs omitted.

None of these results demonstrates that there is no place for maintenance chemotherapy for AML, but the relapse-free pattern suggests that it may be superfluous for at least a proportion. The results of other studies also suggesting that the intensity of the initial and immediate post-remission therapy, whether chemotherapy or chemotherapy with radiotherapy and bone marrow transplantation, persuade us to pursue this direction of research.

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Multicentre Pilot Study for Therapy of Acute Lymphoblastic and Acute Undifferentiated Leukemia in Adults*

D. Hoelzer, E. Thiel, H. Löffler, H. Bodenstein, T. Büchner, and D. Messerer,
for the German Multicentre ALL/AUL Study Group

In 1978 a study group was formed in the Federal Republic of Germany for the treatment of acute lymphoblastic leukemia (ALL) und acute undifferentiated leukemia (AUL) in adults. A modified form of an intensive induction and consolidation regimen successful in children with ALL [1] was used. The objective was to determine whether a prolonged remission induction and the addition of the drugs cyclophosphamide, cytosine-arabinoside and 6-mercaptopurine to the more conventional drugs for induction therapy of ALL, vincristine, prednisone, daunorubicin and L-asparaginase would improve the long-term results. It was hoped that a large number of hospitals would participate and that, with a sufficient number of patients uniformly treated, it would be possible to determine prognostic factors and to identify risk groups. This report concerns the design of the study, the toxicity of the therapeutic regimen and the first results.

I. Design of Study

In this multicentre, prospective, non-randomised study, patients between 15 and 65 years of age with ALL or AUL were treated according to the protocol. Excluded from the study were patients with previous intensive chemotherapy or severe pre-existing somatic or psychiatric disease.

II. Diagnostic

The aim was to have central diagnosis for all participating insitutes including morphology and cytochemistry, cell surface marker studies and determination of terminal deoxynucleotidyl transferase (TdT). This central execution of the diagnostic required a considerable amount of organisation and expense and could thus be only partially achieved during the pilot study.

The *morphological* diagnosis was made on the basis of Giemsa staining of blood and bone marrow smears and the cytochemical reactions PAS, peroxidase, naphthyl acetate esterase. To avoid delay, therapy was commenced according to the diagnosis made locally and, in addition, blood and bone marrow smears were stained and reviewed in one centre (H. Löffler, Kiel). Considered as ALL were cases with PAS-positive blasts and as AUL cases with blasts negative in all cytochemical reactions and without signs of granulopoietic differentiation [2].

The *immunological* diagnosis by cell surface marker analysis of blood and bone marrow cells was made in Munich (E. Thiel) for all participating institutions. According to methods already described [3], ALL should be classified into subtypes (c-ALL, c/T-ALL, pre-T-ALL, T-ALL, B-ALL) and morphological AUL identified either as a subtype of ALL, as unclassifiable "Null"-ALL or possibly as myeloid or erythroid leukemia. Furthermore, the enzyme terminal deoxynucleotidyl transferase was determined centrally by a biochemical and immunofluorescence assay (H. Bodenstein, Hannover).

* Supported by the Bundesministerium für Forschung und Technologie, Förderungs Nr. 01 ZW 450

III. Therapy

The treatment comprises induction therapy with CNS-prophylaxis, consolidation therapy and continuous maintenance therapy over 2 years. The 8-week *induction* regime (Fig. 1) consists of two phases: *Phase I* prednisone, 60 mg/m² p.o., days 1–28, vincristine 1.5 mg/m² i.v. weekly, days 1, 8, 15, 22, daunorubicin 25 mg/m² i.v. weekly, days 1, 8, 15, 22, L-asparaginase 5,000 u/m² i.v., days 1–14; *Phase 2,* cyclophosphamide 650 mg/m² i.v., days 29, 43, 57, cytosine-arabinoside 75 mg/m² i.v. for 4 days × 4, days 31, 38, 45, 52, 6-mercaptopurine 60 mg/m² p.o., days 29–57. The *CNS-prophylaxis,* following achievement of complete remission, consists of methotrexate 10 mg/m² i.th., days, 31, 38, 45, 52 and CNS-irradiation with 24 Gy. A 6-week *consolidation* course (Fig. 2) is given after 3 months similar to the induction regime but with dexamethasone and adriamycin instead of prednisone and daunorubicin and without L-asparaginase. *Maintenance* therapy comprises 6-mercaptopurine 60 mg/m² p.o. daily and methotrexate 20 mg/m² p.o. or i.v. weekly for a period of 2 years.

Patients with a leucocyte count over 25,000 cells/µl and/or large tumour masses may receive a pre-phase therapy with vincristine and prednisone. A 1- to 2-week interval may be inserted between phase 1 and phase 2 of the induction therapy when the blood values or the clinical status require it. Since it was expected that drug toxicity would be higher for adolescents and adults than for children, the induction therapy of the West Berlin scheme was modified by reducing L-asparaginase in dose and duration and the other drugs in dose. In the consolidation therapy, L-asparaginase was omitted altogether as was also the intrathecal methotrexate and the dose reduced for the other drugs. Furthermore, in view of the greater risk of bleeding, particularly of cerebral haemorrhage, in patients over 35 years, it was recommended

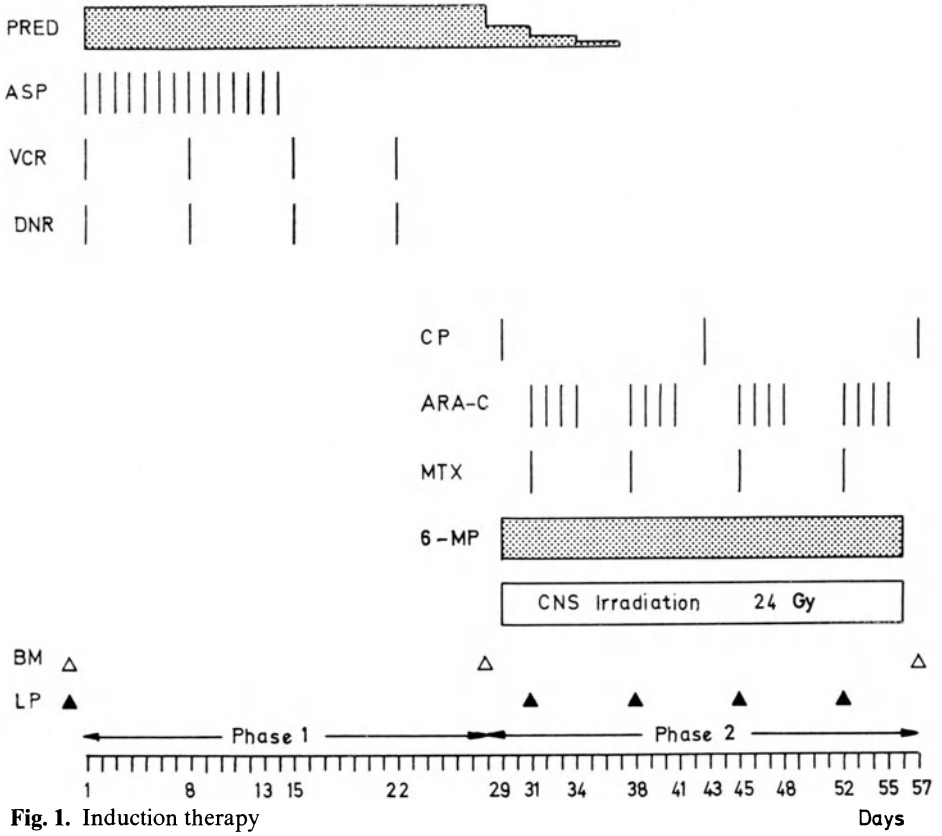

Fig. 1. Induction therapy

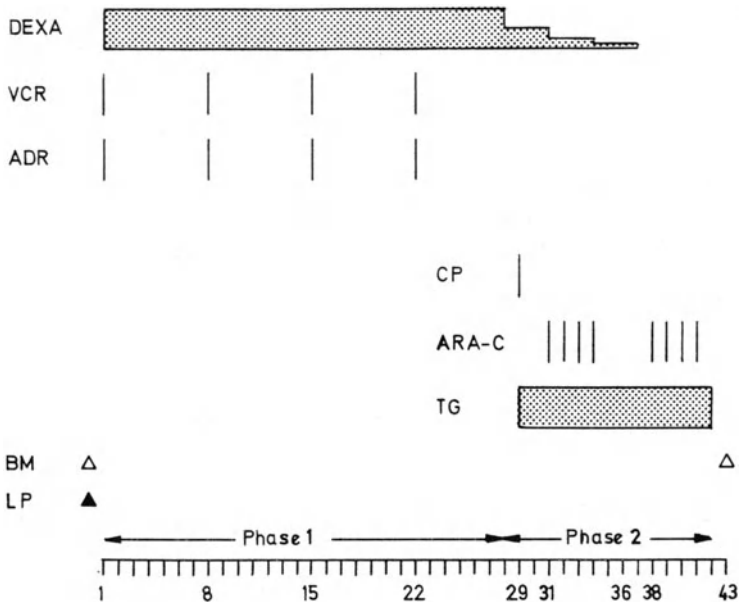

Fig. 2. Consolidation therapy

that in the induction therapy L-asparaginase should only be started when platelets were above 50,000/μl following the initial therapy. In addition, the dose of drugs in phase 2 was reduced to 2/3 in patients of this age group.

IV. Statistical Evaluation

Collection of data and its statistical evaluation was carried out at the Biometrical Centre for Therapy Studies (D. Messerer and T. Zwingers) in Munich. To assess therapeutic results, bone marrow samples were taken after phase 1 and phase 2 of the induction therapy (Fig. 1). Complete remission (CR) rate was the percentage of patients in complete remission after phase 2 of induction therapy, remission duration was timed from the first M_0 or M_1 bone marrow to relapse, death or last follow-up in CR and survival time from diagnosis to death or last follow-up. The Kaplan-Meier [4] method was used to calculate curves for remission duration and survival time.

V. Recruitment of Patients

Participation in the therapy study was open to all hospitals in the Federal Republic of Germany that had sufficient experience in the treatment of malignant disease and the means to provide adequate supportive therapy. The pilot study started in October 1978 and closed at the end of June 1981. During this time, 170 patients from 25 hospitals were recruited, 162 of whom were eligible. The closing date for this analysis was May 30, 1982.

The main phase was activated on July 1, 1981, and up to May 30, 1982, 101 additional patients accrued but are not considered in this evaluation.

A. Results

A complete remission was attained by 77.8% (126/162) of the patients, a partial remission by 3.7% and 18.5% were failures

Table 1. Results of induction therapy

		CR	PR	F
Total		77.8% (126)	3.7% (6)	18.5% (30)
Age	≦35 J.	81.0%	3.3%	15.7%
	>35 J.	68.3%	4.9%	26.8%
Diagnosis	ALL	77.3%	4.1%	18.6%
	AUL	78.5%	3.2%	18.5%

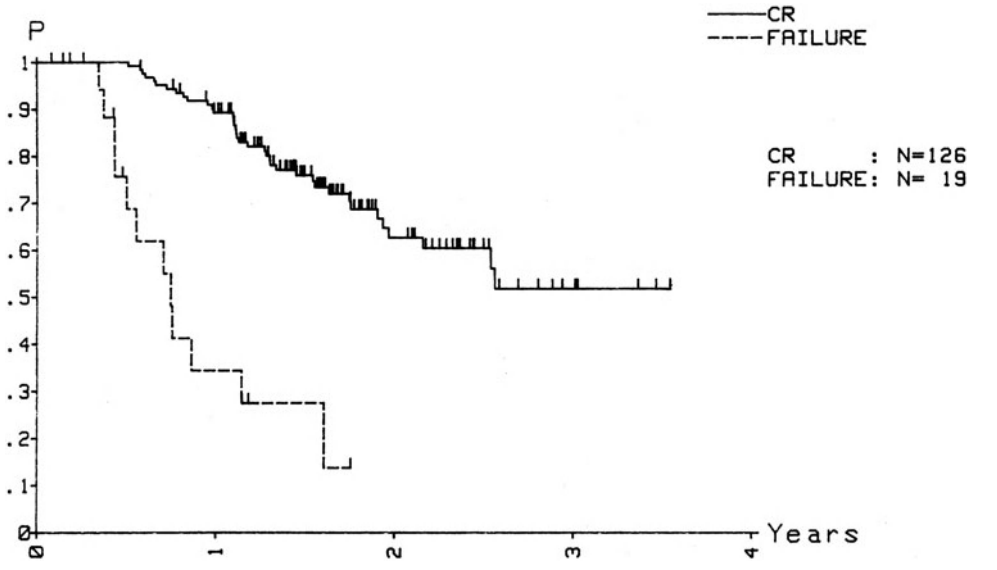

Fig. 3. Survival time (cumulative proportion alive) of patients with complete remission or failure to respond

(Table 1). The complete remission rate for patients under 35 years was 81.0%, for those over 35 years 68.3%. Regarding the morphological and cytochemical diagnosis of ALL or AUL, there was no difference between the remission rates of 77.3% and 78.5%. The median duration of remission for the 126 patients who reached CR was 20 months (confidence limits, 19–21 months). The median survival time for all 145 patients who completed the induction therapy was 31 months. For the 126 patients with CR, the median survival time (Fig. 3) has not yet been reached (last observation 43 months); for the non-responders (without death during the induction period) the median was 9 months.

Table 2. Toxicity during induction therapy

	Moderate	Severe
Infection	17%	9%
Sepsis	7%	5%
Haemorrhage	6%	6%
Stomatitis	1%	–
Gastrointestinal side effects	9%	2%
Hepatotoxicity	11%	2%
Pulmonary toxicity	1%	1%
Cardiotoxicity	1%	–
Neurotoxicity	8%	1%
Psychosis (steroid)	3%	–
Other	7%	6%

I. Toxicity

The frequency of moderate and severe toxicity is given in Table 2. The most common complications were infection, including sepsis, and haemorrhage. Gastrointestinal side effects and hepatotoxicity were most probably drug related as well as the neurotoxicity induced by vincristine. Pulmonary toxicity and cardiotoxicity were seldom observed. Seventeen (10.5%) of the 162 patients died during induction therapy. The most frequent causes of death were haemorrhage, particularly cerebral and gastrointestinal, and resistant infections, particularly pneumonia and sepsis. Death occurred mainly (15/17) in phase 1 and correspondingly seldom (2/17) in phase 2 of the induction therapy.

B. Discussion

The observation time for this study was too short to provide any conclusive information on the long-term results of the intensified induction and consolidation regimen. However, a reasonable number of 170 ALL/

AUL patients could be recruited and together with the 101 patients from the first 11 months of the main phase study a total of 271 patients entered the trial, which is a substantial proportion of patients with that disease in the Federal Republic of Germany.

The success of the intensified induction regime is demonstrated by the complete remission rate of 77.8%, which is relatively high compared to other multicentre trials (literature summarised by Esterhay et al. [5]), especially when the large number of participating institutes is considered. A better response rate was seen for younger patients as in other trials. However, except for hepatomegaly, no other clinical and laboratory features had any statistically significant influence on the achievement of complete remission. Also for the morphologically defined acute lymphoblastic and acute undifferentiated leukaemia the CR rate was very similar.

The protocol seems tolerable with regard to toxicity and manageable even in patients over 35–65 years. The main complications were, as in other studies, bleeding and infection. Pulmonary toxicity and cardiotoxicity were seldom seen, the latter probably because of the low dosage of daunorubicin. The frequency of death during induction is of a similar order as in other adult ALL trials, and the main causes of death were bleeding and infection. Since only two patients died during the intensive phase 2 of the induction therapy with the drugs cyclophosphamide, cytosine-arabinoside and 6-mercaptopurine, whereas the majority of patients died in phase I, it seems reasonable to assume that death was due not only to drug toxicity but also to the advanced stage of the disease.

Regarding the long-term results, the median remission duration (MRD) of 20 months (confidence limits, 19–21 months) is one of the longest observed in large trials [5], apart from the superior results in a monocentric study where the very intensive L10/L10M protocol was used [6]. The survival time for the complete responders reflects the MRD. Preliminary analysis gave as possible prognostic factors: the time needed to achieve CR (i.e. the number of therapy courses required), age, initial leucocyte count and the immunological subtype. The observation time was, however, as yet too short to provide a reliable assessment of their significance.

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Haematology and Blood Transfusion Vol. 28
Modern Trends in Human Leukemia V
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The Treatment of Acute Myelogenous Leukemia in Children and Adults: VAPA Update*

H. J. Weinstein, R. J. Mayer, D. S. Rosenthal, F. S. Coral, B. M. Camitta, R. D. Gelber, D. G. Nathan, and E. Frei, III

A. Introduction

Major progress in the treatment of acute myelogenous leukemia (AML) has occurred during the past decade. Advances in chemotherapy and supportive care have been associated with an increase in the complete remission rate of patients under the age of 60 with AML from 35%–55% [5, 7] to approximately 75% [2, 9, 16]. More importantly, the median duration of complete remission and the percentage of patients in long-term continuous complete remission has steadily improved. This has resulted from postinduction combination chemotherapy [10, 13, 18, 20] or chemoradiotherapy and transplantation of marrow from histocompatible siblings [4, 15, 21]. In 1976 the VAPA protocol was initiated to specifically improve the duration of complete remission for children and adults (< 50 years) with AML. In 1980 we reported encouraging results obtained with this approach [12, 22], and this report is an update of the study.

B. Materials and Methods

I. Patients

One hundred and seven consecutive, previously untreated patients less than 50 years of age were evaluated and entered onto this study between February 1976 and October 1979. The diagnosis of AML was based on morphologic examination of bone marrow and a study of histochemical stains.

II. Treatment

Remission was induced with two courses of vincristine, adriamycin, prednisolone, and cytosine asrabinoside (araC). Patients achieving complete remission were treated with intensive sequential combination chemotherapy for 14 months. This phase of therapy was divided into four sequences of drug combinations: adriamycin/araC, adriamycin/azacytidine, prednisolone/vincristine/mercaptopurine/methotrexate, and araC. Central nervous system prophylaxis was not included but surveillance lumbar punctures were performed throughout remission. Details of the treatment protocol have previously been published [22].

III. Statistical Analysis

The duration of survival was measured from the time of initial therapy while the duration of remission extended from the time bone marrow remission was confirmed. Kaplan-Meier analyses were performed for survival and continuous complete remission. Statistical tests on this distribution were made with the log-rank test [14]. Deaths during remission were treated as relapses and withdrawals were considered up until the time they were electively removed from the protocol.

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C. Results

I. Induction of Remission

The results of remission induction therapy are presented in Table 1. Rates of complete remission were similar for children and adults and did not differ significantly according to morphologic subtype of AML (data not shown).

II. Duration of Remission

Among the 75 complete responders there have been eight withdrawals for reasons including nonhematopoietic drug toxicity, bone marrow transplantation, and physician-patient desire to discontinue therapy. There have been two deaths during remission in the adult group. A total of 34 patients have relapsed. Eight of 19 pediatric relapses have occurred in the central nervous system (CNS). In contrast, only one of the relapses in the adult group occurred in the CNS (Table 1).

Figure 1 is a Kaplan-Meier plot of the probability of remaining in continuous complete remission (CCR). The median follow-up period for patients less than age 18 is 41 months and 52% remain in CCR at 38 months. For patients age 18–50 the median follow-up period is 33 months, and there is a 42% probability of CCR at 36 months but this decreases to 14% at 51 months. This late fall in the adult curve is a reflection of the small number of adults at risk beyond 3 years and two late relapses.

Table 1. VAPA update as of 1 April 1982

	Age 0 – 17	Age 18 – 50
No. entered	61	46
No. of complete remissions	45 (74%)	30 (65%)
Withdrawals	5	3
Deaths in remission	0	2
Total relapses	19	15
CNS	8	1
Bone marrow	10	13
Myeloblastoma	1	1
Completed therapy	26	15
Relapses of therapy	5	5

III. Cessation of Treatment

Twenty-six patients less than 18 years of age have completed therapy and only 5 of these 26 patients have relapsed (median follow-up time after cessation is 21+ months). Fifteen adults have completed treatment and five have relapsed (median follow-up after cessation is 18+ months).

IV. Overall Survival

Figure 2 is a Kaplan-Meier plot of the probability of survival for all patients: the median survival remains undefined for patients less than 18 years of age and the median survival for patients 18–50 years of age is 16.5 months.

V. Prognostic Factors

Factors that may have influenced the duration of remission were analyzed using the log-rank test. These factors included white blood count at the time of diagnosis, age, sex, morphologic subtype of AML, and the number of courses of therapy required to induce a complete remission. The monocytic subtype was the only presenting feature that correlated significantly with remission duration. Patients less than 18 years of age with monocytic leukemia had shorter lengths of complete remission ($P = 0.007$).

VI. Central Nervous System Relapse

Seven of the eight children with primary CNS relapse were asymptomatic. All eight children had bone marrow relapses 2 weeks to 5 months after CNS relapse. The monocytic subtype was associated with a high risk for primary CNS relapse ($P = 0.07$).

VII. Toxicity

Toxic manifestations during the intensive sequential chemotherapy phase were limited primarily to nausea, vomiting, and fe-

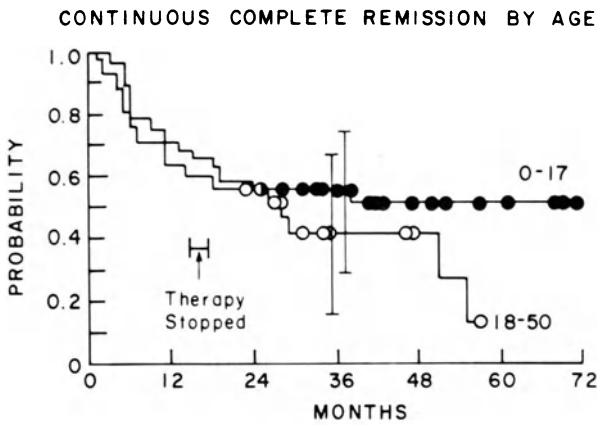
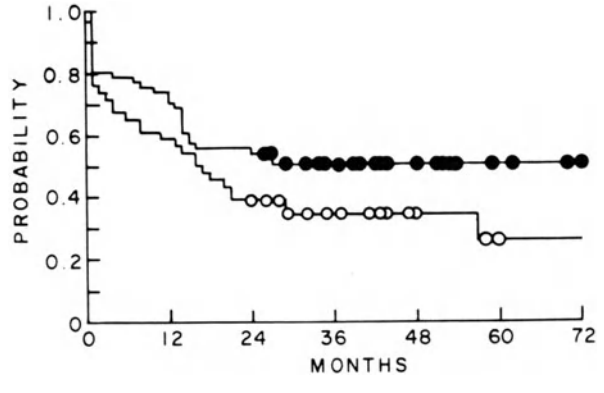


Fig. 1. Kaplan-Meier plot of probability of CCR. The *vertical bars* represent two standard deviation confidence limits



AGE	ALIVE	DEAD	TOTAL	MEDIAN
● 0-17 YRS	31	30	61	UNDEF
○ 18-50 YRS	15	31	46	16.5

Fig. 2. Kaplan-Meier plot of probability of survival for all patients ($n = 107$)

ver (or infection) associated with granulocytopenia. The average hospital time during this phase of therapy was approximately 60 days. After 1978, patients received continuous subcutaneous infusions of araC outside the hospital by means of a portable infusion pump (autosyringe) instead of continuous intravenous infusion as inpatients. Three of 75 patients followed in remission developed adriamycin cardiomyopathy, with one fatality in this group. There was one death during remission secondary to pneumonia during a period of granulocytopenia. There was a 5% incidence of reversible cholestatic liver disease observed during the maintenance phase.

D. Discussion

In this study, 70% of patients with AML entered complete remission. This result is consistent with the experience of others who have employed a combination of cytosine arabinoside and an anthracycline with or without vincristine and prednisolone. The goal of our study was to improve durations of complete remission by specifically addressing the problem of relapse. The VAPA protocol included 14 months of intensive sequential chemotherapy after remission was achieved. The programm was designed to maximize leukemic cytoreduction and to circumvent the problem of

the development of drug resistance by leukemia cells [19].

The overall data appear very encouraging. In the pediatric group, the probability of CCR is 56% at 2 years, and only one relapse has been observed beyond this time. Twenty-six of 45 children who entered complete remission had therapy electively stopped at 14 months in CCR and 21 of these patients remain in remission. These results are significantly better from those reported in other chemotherapy trials for childhood AML [2, 6, 8].

For adults between 18 and 50 years of age, the median duration of remission was 28 months, which is substantially longer than previously achieved with most other chemotherapy protocols [1, 11]. There have been, however, two late relapses (after 3 years of CCR) in the adult group. Due to the small number of patients at risk beyond 3 years, one cannot predict with certainty the percentage of adults who will remain in long-term remission. Preliminary results of other intensive chemotherapy programms for adults with AML support our treatment approach [3, 17].

The central nervous system was the initial site of leukemic relapse in 8 of 19 children. In contrast, only one adult experienced a CNS failure amongst 15 adult relapses. Primary CNS relapse has been reported to account for 10%–15% of the relapses in both children and adults with AML [8, 24]. The VAPA protocol did not incude CNS prophylaxis, but cytosine arabinoside penetrates into the CSF when administered by continuous intravenous or subcutaneous infusion [23]. The high incidence of primary CNS relapse in children indicated that continuous araC infusions at a dose of 200 mg/m^2 per day were not effective for CNS prophylaxis. There were many more children than adults with monocytic leukemia in our study and this may have contributed to the higher CNS relapse rate observed in the younger group. In our new AML study patients less than 18 years of age receive intrathecal chemotherapy for CNS prophylaxis.

The VAPA experience indicates that AML, especially in children, can be controlled and hopefully cured by chemotherapy alone in many patients. The only other therapy that appears to maintain long durations of remission for patients with AML is chemoradiotherapy followed by allogeneic bone marrow transplantation performed early in the first remission. This approach is currently limited to patients with a histocompatible sibling. With continued advances in supportive care, chemotherapy, and bone marrow transplantation, long-term control of AML in the majority of cases is a reasonable goal in the near future.

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Improved Results in Treatment of Acute Myelogenous Leukemia in Children – Report of the German Cooperative AML Study BFM-78*

U. Creutzig, G. Schellong, J. Ritter, A. H. Sutor, H. Riehm, H. J. Langermann, A. Jobke, and H. Kabisch

Recently the treatment programs for childhood acute myeloid leukemia (AML) have become more effective, not only in achieving a higher percentage of induction responses but also in the improvement of duration of the first remission [7, 9, 12]. Because AML in children is rare – about 80 new cases per year are expected in West Germany and West Berlin – it is necessary to cooperate in multicenter trials to gain experience and to establish the value of new therapies.

A. Patients and Methods

Between December 1978 and May 1982, 138 children aged less than 17 years with AML entered the protocol of the childhood AML study BFM 78, which is a cooperative prospective trial of 31 German hospitals.

The initial treatment consists of an induction regimen (Fig. 1) with two intensive 4-week cycles, which resembles in design the West Berlin ALL Protocol [8]. Before starting the protocol reduction of the cell mass in patients with high leukocyte counts is attempted by using low doses of 6-thioguanine (6-TG) per os and cytosine arabinoside (ara-C) i.v. daily. Prednisone therapy should not be started as long as the danger of severe bleeding exists.

Seven different drugs and prophylactic irradiation to the skull are given over a period of 2½ months, with a recovery period of 1–2 weeks between phase 1 and 2. Maintenance therapy lasts for 2 years. 6-TG (40 mg/m² per os) is given daily and ara-C (40 mg/m² s.c.) every 4 weeks for 4 days and in addition adriamycin (Adr) (25 mg/m² i.v.) every 8 weeks but only in the first year. The diagnosis AML is based upon the morphological and cytochemical criteria of the FAB classification [2].

Patients who failed to achieve complete remission (CR) after the induction regimen were classified as nonresponders. Relapse was diagnosed on appearence of more than 5% of blasts in the bone marrow or of leukemic cells at any other site. Methods of

Table 1. Characteristics of 138 protocol patients of the AML therapy study

	n	(%)
Total	138	
Boys	73	(53)
Initial CNS involvement	10	(8)
Initial extraordinary organ manifestation	26	(19)
Liver ≧ 5 cm below costal margin	33	(24)
Spleen ≧ 5 cm below costal margin	36	(27)
Leukocyte count $< 5 \times 10^9$/liter	28	(21)
Leukocyte count $> 100 \times 10^9$/liter	31	(23)
Platelet count $< 10 \times 10^9$/liter	16	(12)
Platelet count $10 - 20 \times 10^9$/liter	23	(17)
Hemoglobin <60 g/liter	18	(13)

* Supported by the Minister for Research and Technology of the Federal Republic of Germany

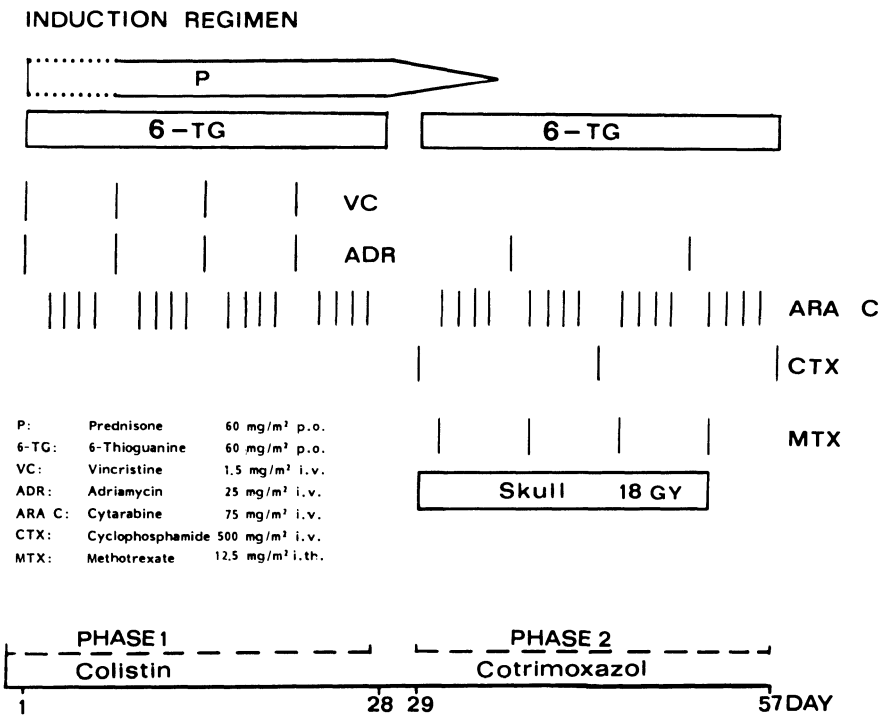

Fig. 1. Induction regimen of the cooperative AML study BFM-78

statistical analysis were the life table algorithm [5] and Cox's regression model [4]. Date of analysis was 1 May 1982.

Patient characteristics are given in Table 1. The median age is 9.8 years. Initial extraordinary organ manifestations were mainly skin involvement or infiltration of the tonsils, submandibular glands, or testes. The classification according to the FAB criteria is:

M 1 and M 2	acute myeloblastic leukemia	(AMBL):	64 (46%)
M 3	acute promyelocytic leukemia	(APL):	6 (4%)
M 4	acute myelomonocytic leukemia	(AMML):	37 (27%)
M 5	acute monoblastic-monocytic leukemia	(AMOL):	28 (20%)
M 6	acute erythroleukemia	(EL):	3 (2%)

B. Results

The results after 41 months are shown in Table 2. Seventy-eight percent of the children achieved CR during induction regimen in median after 43 days. Thirteen children died of cerebral bleeding within the first 12 days, two of them before onset of therapy. Five more patients died of therapy complications or infections and six children died later on in remission. Twelve patients (9%) were classified as nonresponders; two of them achieved CR after starting maintenance therapy. For sites of relapses see Table 2. Twenty-four of the total of 33 relapses occurred in the bone marrow only. There was only one isolated CNS relapse.

Sixty-seven children have been in continuous complete remission (CCR) for 1–41 months. In addition, two children have been in first remission for 2 years, although further treatment was refused after the induction regimen. The life table analysis indicates that the probability for CCR for the total group of 138 patients after 41 months is 0.40 ± 0.05 (Fig. 2). The

probability of CCR for the remission group (nonresponders and early deaths during induction therapy are excluded) is 0.57 ± 0.07 after 41 months. Death during remission induction as well as poor response to therapy occurred more frequently in AMML and AMOL patients. Long-term results are not significantly different among these subgroups.

The analysis of risk factors indicates that fatal hemorrhage is more common in AMOL patients than in other subgroups (descriptive P value $= 0.009$). The analysis of hemostasiological factors in 34 patients showed that the plasminogen levels were low in those five patients who died of early cerebral bleeding, whereas the other patients had normal plasminogen levels [11]. In general the risk of relapse is not predictable. Only in the subgroup of AMML do initial extraordinary organ manifestation ($P = 0.03$) and high initial leukocyte count

Table 2. Treatment results in 138 patients of the AML therapy study BFM-78

	n		
Patients	138		
Death before onset of therapy	2		
Death during induction therapy			
Bleeding	11		
Infection	3		
Therapy	2		
Nonresponders	12		
Complete remission achieved	108 (78%)	*Relapse sites*	
Death in remission	6	BM (bone marrow)	24
Relapses	33	CNS	1
In CCR (1 – 41 month)	67	Testes	1
Alive	79	Skin	2
		BM/CNS	3
		BM/testes	2

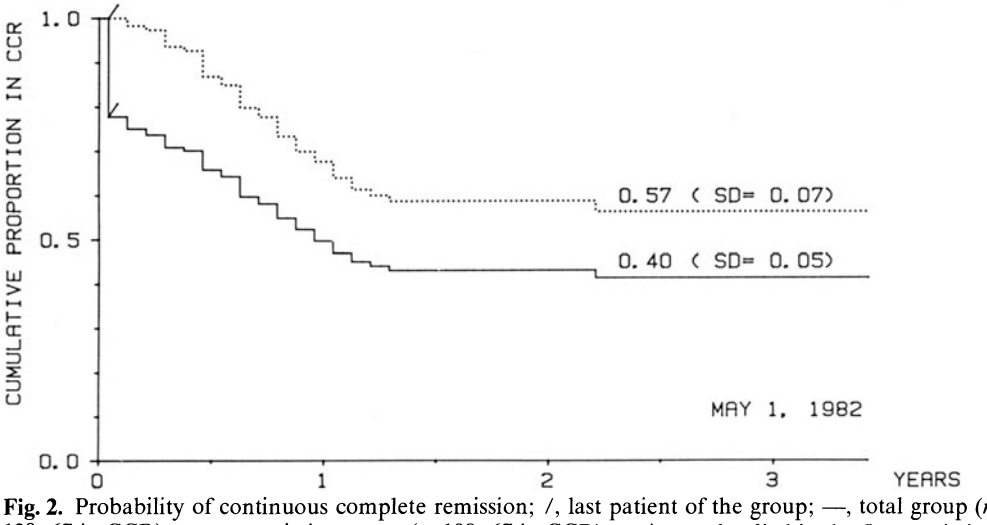

Fig. 2. Probability of continuous complete remission; /, last patient of the group; —, total group (*n*, 138; 67 in CCR). · · · · ·, remission group (*n*, 108; 67 in CCR); patients who died in the first remission were censored at the date of expiry

($P=0.01$) correlate with increased risk of relapse.

C. Discussion and Conclusions

The preliminary results of the German AML study BFM 78 make the following conclusions possible:

1. A prolonged intensive induction therapy using seven drugs and preventive cranial irradiation produces a high proportion of remission (78%) in childhood AML and – in combination with a continuous maintenance therapy over a period of 2 years – a 57% rate of CCR after 3.5 years.

2. The results of the German AML study are in general comparable to those of the VAPA-10 study in Boston, concerning only the children [12]. However, the localization of recurrences is markedly different. In the BFM study only 1/33 relapses was an isolated CNS infiltration (in a patient with initial CNS involvement) compared to 7/14 in the VAPA-10 study (children only). Therefore we conclude that prophylactic cranial irradiation has become a very important factor in childhood AML, since the duration of the remission period has been prolonged considerably.

3. In contrast to childhood ALL, high initial leukocyte counts generally do not seem to be a risk factor for recurrences with BFM therapy. Only the combination of AMML with large tumor mass, which is more frequent in this subgroup than in the others, indicates a high risk of relapse.

4. It is remarkable that the portion of children with AMOL in the BFM study (20%) is higher than in adults. Sultan et al. [10] found 31/250 adult patients with AMOL (12%) and Economopoulos et al. [6] 8/75 (11%). So far only few data exist on the percentage of AMOL patients in childhood AML. The range is 9%–16% ([1, 3]; Weinstein, personal communication at Wilsede Joint Meeting 1982).

There are no reports of other pediatric groups about early fatal bleeding in AMOL subtype. Chessels et al. [3] found that early death from leukostasis occurred in AMML and AMOL patients. In the VAPA-10 study the reason for the low incidence of fatal cerebral bleeding (1/61) might be the effectiveness of therapy with hydroxyurea before starting the protocol. Our analysis of hemostasiological factors has shown a low plasminogen level even before onset of bleeding in those children who died of cerebral hemorrhages. Future efforts should be directed toward avoiding this complication in AML children, especially in those with AMOL.

5. In general the results of the German AML study as well as the data of the VAPA-10 study support the hope that childhood AML will no longer be an incurable disease if treated with suitable aggressive chemotherapy programs.

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Acute Myeloid Leukaemia in Childhood: Treatment in the United Kingdom

J. M. Chessells, C. A. Sieff, and A. Rankin

Childhood acute myeloid leukaemia (AML) is rare. The annual incidence rate, based on the Manchester Children's Tumour Register [1], is 5.1 cases per 10^6 children under 15 years of age, thus approximating 60–70 new cases per annum in the United Kingdom. At the Hospital for Sick Children, Gt. Ormond Street (HSC), an average of eight to ten new cases of AML are referred per annum.

A. Treatment Before 1975

Figure 1 shows the survival of all patients treated in two consecutive periods at the HSC. Before 1972 patients received a variety of drugs; between 1972 and 1975 daunorubicin and cytosine arabinoside (araC) were used in induction, CNS prophylaxis was given with intrathecal chemotherapy and treatment continued with 6-mercaptopurine or thioguanine for 2 years. There was no improvement in median survival but three patients remain in remission over 8 years from diagnosis.

B. The MRC UKAML Trial

Between 1975 and 1979 patients at the HSC were entered into the first Medical Research Council (MRC) trial for childhood myeloid leukaemia (UKAML). The design of this multicentre trial is shown in Fig. 2. Induction chemotherapy was followed by consolidation and CNS prophylaxis. At first patients were randomized to receive either no further treatment after consolidation, or immunotherapy. Immunotherapy consisted of BCG and irradiated allogeneic blast cells; one dose of 0.2 ml containing BCG and 2.5×10^7 blast cells was given intradermally each month for four doses. Subsequently a third arm was introduced, comprising 2 years maintenance chemotherapy with daily oral thioguanine and weekly subcutaneous araC.

I. Remission Induction

One hundred and fifty-eight patients were entered from centres throughout the United Kingdom. One hundred and five of these (66%) achieved remission after a median of three courses of chemotherapy. The cause of failure to achieve remission was analysed as suggested by Preisler [2]. Using his criteria 35 patients would be classified as class I or II failures who by implication might have benefited from more intensive chemotherapy. Early deaths (type V failures) accounted for 13 failures; six of these were associated with haemorrhage with or without disseminated intravascular coagulation, six with leucostasis and only one with infection.

Analysis of response to initial chemotherapy in relation to FAB class as de-

Table 1. UKAML response to induction according to FAB class

	M_1	M_2	M_3	M_4	M_5	M_6
No.	20	60	8	34	26	9
CR	13	46	2	25	17	1
Prop.	0.65	0.76	0.25	0.73	0.65	0.1

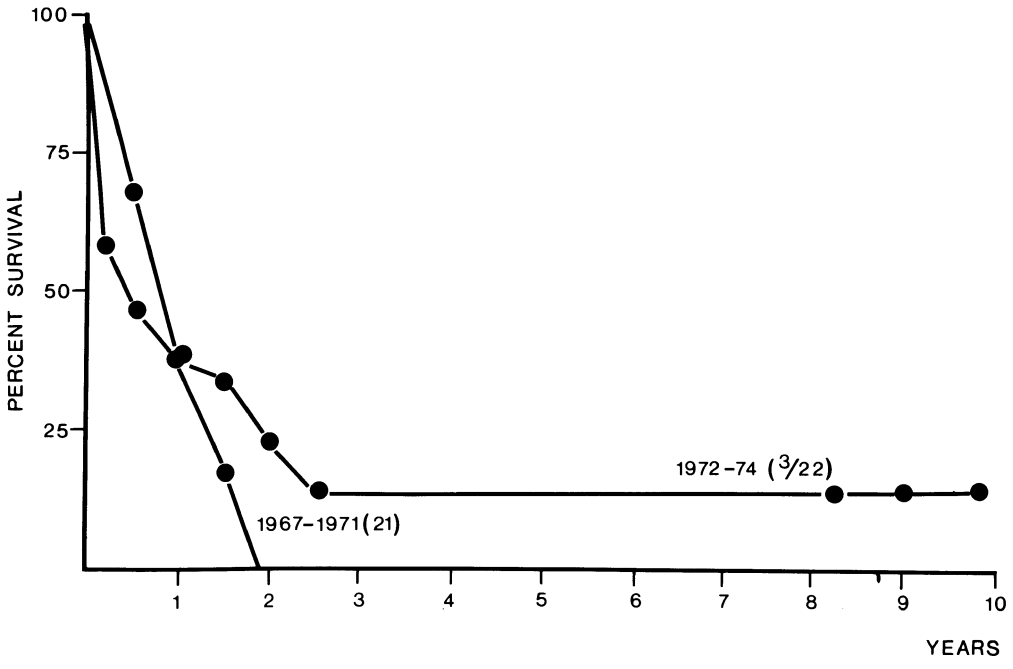

Fig. 1. Life table analysis of survival in children with AML treated in two consecutive periods at the HSC. Numbers in brackets indicate number still alive/total number

scribed by Bennett et al. [3] (Table 1) shows that the worse response is in M_3 AML, because of death from haemorrhage, and in M_6 AML because of failure to respond to chemotherapy. The deaths from leucostasis occurred in patients with M_4 and M_5 AML. The overall remission rate was lowest (52%) in patients with initial leucocyte counts in excess of 100×10^9/litre and highest (72%) in those with counts under 10×10^9/litre.

II. Consolidation

Consolidation chemotherapy comprised six courses of adriamycin, araC, thioguanine and vincristine and six doses of intrathecal araC. Cranial irradiation was not given. The overall incidence of CNS relapse was low with CNS relapse as a first event occurring in only three patients. Seventeen patients (11%) relapsed during this consolidation phase so that the overall number of patients available for randomization was 88 (56%). Four of these patients were not randomized, receiving transplantation or alternative chemotherapy, and have been withdrawn from subsequent analysis.

III. Further Treatment

The outcome in patients receiving no further therapy, immunotherapy and maintenance treatment is shown in Fig. 3. Although these results may appear to show

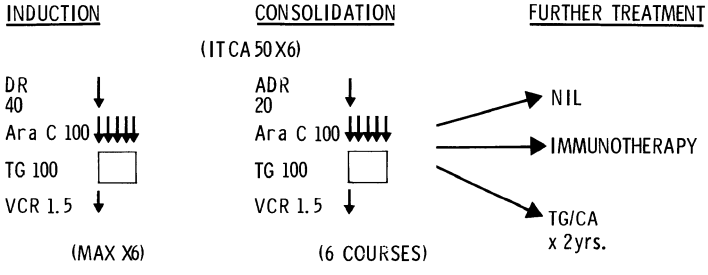

Fig. 2. Design of the MRC UKAML trial 1975–1979 (Drug dosages in mg/m² surface area)

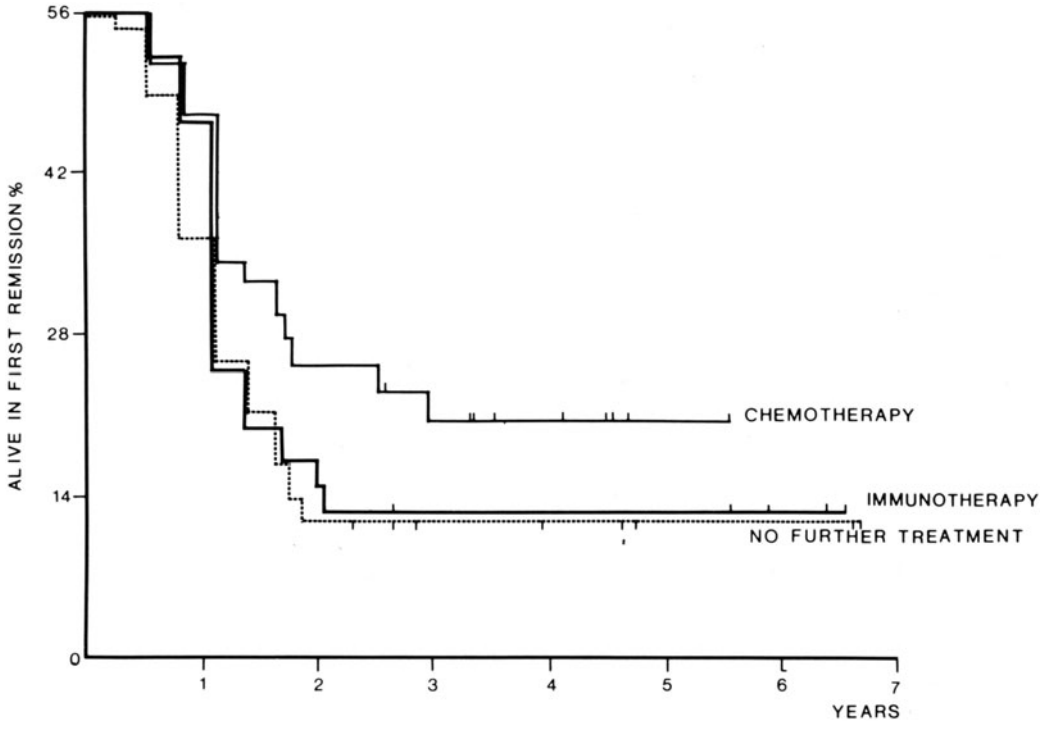

Fig. 3. MRC UKAML trial. Comparison of first remission duration in patients receiving chemotherapy, immunotherapy or no further treatment

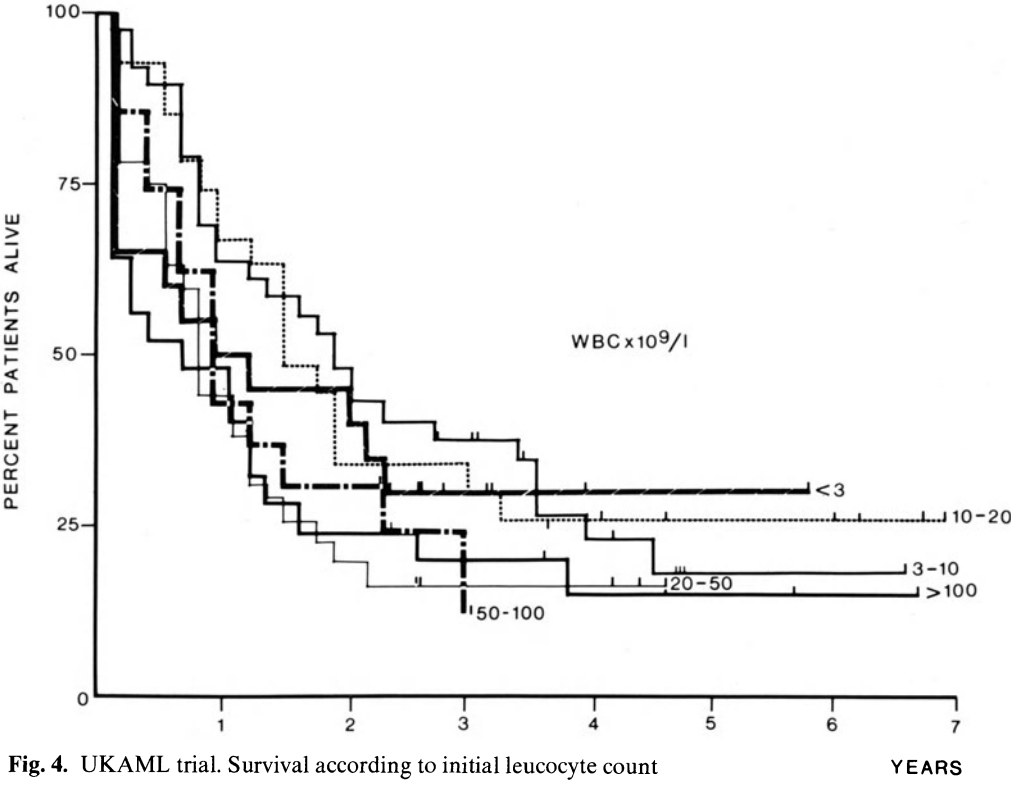

Fig. 4. UKAML trial. Survival according to initial leucocyte count

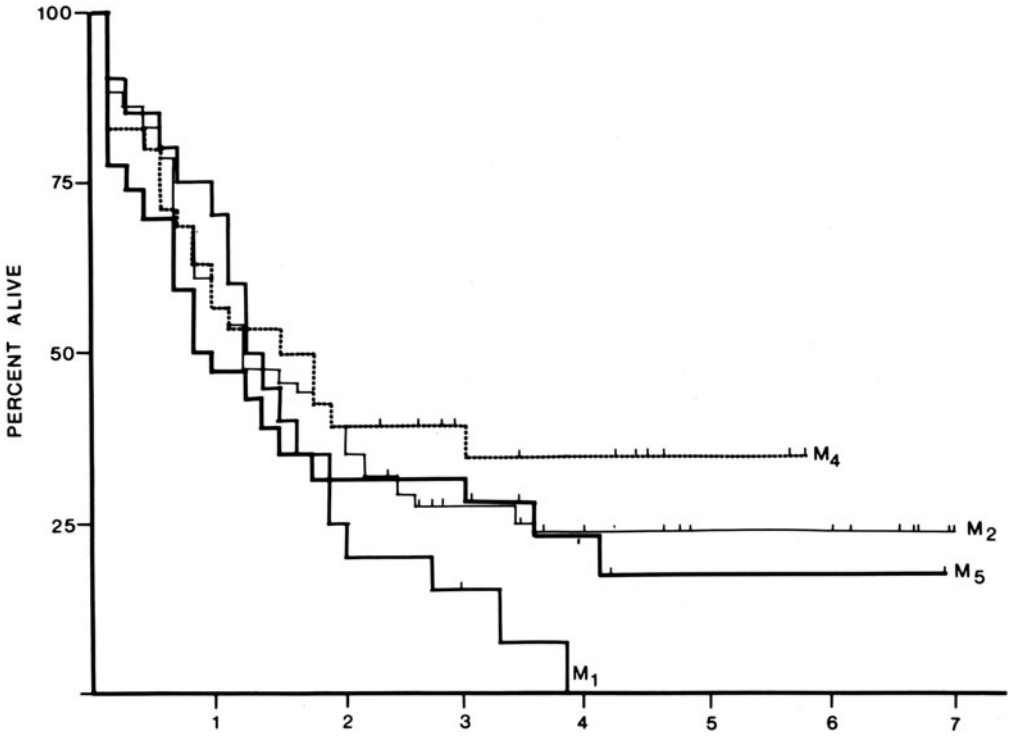

Fig. 5. UKAML trial. Survival according to FAB subtype

an overall advantage for chemotherapy, the comparison with patients randomized later does not show this effect and it appears that continued chemotherapy is at most of marginal benefit.

Analysis of results in terms of leucocyte count at presentation (Fig. 4) shows no consistent relationship but analysis of FAB class and survival (Fig. 5) suggests that patients with M_4 AML and M_2 AML may have a better prognosis than the other subtypes. There was no marked influence of age or sex on prognosis.

IV. Conclusions

This trial illustrates the difficulties arising in accruing patients with a rare disease for a randomized trial. In retrospect, as expected, immunotherapy proved of no benefit in maintaining disease-free survival and long-term chemotherapy was of marginal, if any, benefit.

V. Bone Marrow Transplantation (BMT) in First Remission

During the latter years of this trial BMT had become increasingly accepted as treatment for AML in first remission. A comparison of patients at the HSC with AML achieving stable remission and treated with chemotherapy compared with patients referred to the Royal Marsden Hospital for BMT in first remission has shown a clear benefit in favour of transplantation [4].

However, a major limitation of this form of treatment, in view of the small family size in the United Kingdom, is the limited number of patients with suitable donors. Less than one-quarter of children with AML seen at the HSC have HLA-DR identical donors.

C. The Eighth AML (Paediatric) Trial

Since there is little evidence that childhood AML is different from adult AML, from

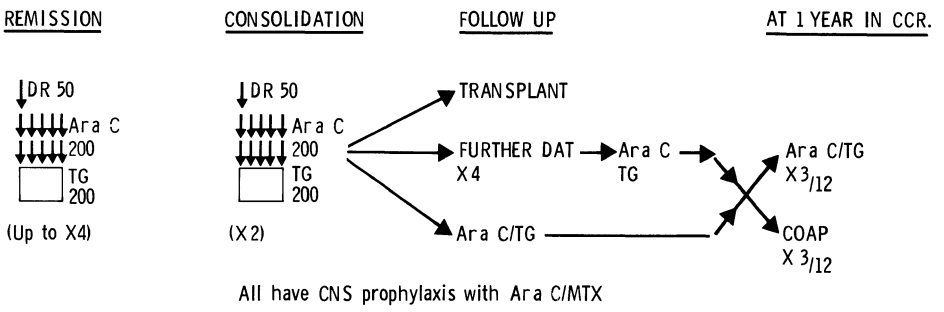

Fig. 6. Design of the MRC eighth AML (paediatric) trial opened May 1980

1980 onwards children have been entered into the ongoing MRC multicentre trial for adult AML. An outline of this protocol is given in Fig. 6. Remission induction is based on the DAT protocol described by Rees and his colleagues at Cambridge [5]. It might be expected that this more intensive regime with 12-hourly araC and thioguanine would produce a higher remission rate than seen in UKAML, and so far 79% of the 48 children entered have achieved remission. Patients in remission receive two further courses of DAT and are then randomized to further consolidation or to start maintenance. All receive chemotherapy for approximately 1 year. Patients with a suitable donor receive BMT in first remission, but so far only four patients have been transplanted.

D. Conclusions

The major obstacle to treatment of AML is bone marrow relapse. At present BMT is only available to the minority of patients with HLA-DR identical donors. It remains to be seen whether more intensive induction and consolidation will increase the proportion of patients achieving disease-free survival or whether developments in BMT will enable this form of treatment to be offered to a larger number of children.

Acknowledgements

We are grateful to the members of the MRC Working Party on childhood leukaemia for permission to quote these data.

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Interferon in Acute Myelogenous Leukaemia: A Preliminary Report

A. Z. S. Rohatiner, F. Balkwill, and T. A. Lister

A. Introduction

Interferon has been shown to have an anti-proliferative effect on leukaemic blast cells in vitro [1, 3, 4]. In short-term liquid culture, 50% inhibition of growth is achieved at IFN-α concentrations of 10^3 units/ml using myeloblasts derived from patients with acute myelogenous leukaemia (AML) [5]. Pharmacokinetic studies in patients receiving IFN-α by continuous intravenous infusion (i.v.i) have shown that it is possible to achieve serum concentrations of 10^3 units/ml at daily doses greater than 30×10^6 units/m^2 [6].

A phase II study of IFN-α is currently in progress at St. Bartholomew's Hospital, to determine whether an anti-proliferative effect can be demonstrated in patients with acute myelogenous leukaemia.

B. Patients and Methods

I. Patients

Fifteen patients with acute myelogenous leukaemia, aged between 31 and 63, are included in this analysis. All had either relapsed following conventional chemotherapy or had demonstrated resistance to therapy comprising adriamycin, cytosine arabinoside and 6-thioguanine. Four patients died whilst receiving IFN-α (two of septicaemia, one of pneumonia and one of cerebral haemorrhage) and are therefore inevaluable.

II. Interferon

IFN-α from a Namalwa lymphoblastoid cell line (Wellcome Research Laboratories) had a specific activity ranging from 2.59×10^7 to 2.13×10^8 units/mg protein.

III. Dosage and Schedule

Between 50 and 200×10^6 units/m^2 of IFN-α were administered continuously for 5,7 or 10 days. Details of dosage and schedule are shown in Table 1.

Table 1. Dosage and schedule

Daily IFN-α dose ($\times 10^6$ units/m^2)	Duration of infusion (days)	No. of patients
50	10	3
100	7	10
200	5	2
Total		15

IV. In Vitro Study

Bone marrow and peripheral blood myeloblasts from patients who subsequently received IFN-α were cultured with IFN-α at concentrations of 10, 10^2, 10^3 and 10^4 units/ml. Growth was assessed at 3 days by viable cell counts under phase-contrast microscopy and uptake of tritiated thymidine.

% ↓ in control cell numbers	Interferon concentrations (units/ml)			
	10	10^2	10^3	10^4
Median	6.2	21.7	58.3	79.8
Range	0 – 18.3	1.6 – 33.4	21.6 – 72.5	31.0 – 88.0
% ↓ in thymidine incorporation	Interferon concentrations (units/ml)			
	10	10^2	10^3	10^4
Median	10.8	25.3	55.6	68.4
Range	0 – 16.3	3.7 – 36.2	17.0 – 58.1	33.2 – 85.0

Table 2. Growth inhibitory effect of IFN-α on myeloblasts in short-term liquid culture

V. Interferon Assay

Serum IFN-α concentrations were measured by reduction of RNA synthesis in V3 cells [2] challenged with Semliki forest virus.

C. Results

I. Anti-proliferative Effect

1. In Vitro

With increasing interferon concentrations, the degree of growth inhibition increased, 50% inhibition being observed at an IFN-α concentration of 10^3 units/ml (Table 2).

2. Clinical Response

Five patients showed no evidence of response. In four patients there was a marked fall in the number of circulating leukaemic blasts but no change in the degree of bone marrow infiltration. In two patients clearing of blasts from the peripheral blood was associated with a decrease in the degree of bone marrow infiltration to less than 5% blasts.

II. Clinical Toxicity

All patients became pyrexial and described symptoms of influenza. Eight patients complained of headache and two of nausea and vomiting. Six patients became drowsy and this symptom persisted for up to a week after the end of the infusion. Two patients receiving IFN-α at a dose of 200×10^6 units/m^2 per day became disorientated and had grand mal fits. In view of this unacceptable CNS toxicity, which was associated with severe biochemical disturbances (see below), subsequent patients received 100×10^6 units/m^2 per day.

III. Haematological and Biochemical Toxicity

All patients became neutropenic and thrombocytopenic. Biochemical evidence of hepatic dysfunction, i.e. transient elevations of alkaline phosphatase and transaminases (SGOT) were observed in 10 out of 11 patients. Reversible hyperkalaemia and hypocalcaemia occurred in two patients receiving 200×10^6 units/m^2.

IV. Serum IFN-α Levels

Peak serum concentrations greater than 10^3 units/ml were achieved in all patients studied.

D. Discussion

Fifteen patients with acute myelogenous leukaemia received IFN-α at doses with resulted in serum concentrations that are inhibitory to myeloblasts in vitro. The clinical toxicity, cytopenia and hepatic dysfunction have previously been described [6].

Myeloblasts from all 15 patients showed evidence of growth inhibition in vitro at IFN-α concentrations which were subsequently acieved in the serum. This was not paralleled in vivo, only 2 of 11 evalu-

able patients showing any decrease in the degree of bone marrow infiltration. There was thus no correlation between the in vitro findings and clinical response.

These preliminary results suggest that IFN-α may have some activity in acute myelogenous leukaemia, and further patients are being entered into the study.

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Human Interferon-Beta in the Treatment of Non-Hodgkin Lymphoma *

W. Siegert, D. Huhn, H. Theml, U. Fink, P. Kaudewitz, G. Riethmüller, and W. Wilmanns

A. Introduction

Interferon has potent antiviral, antiproliferative, and immunomodulating properties [9, 10]. Leukocyte and fibroblast interferon have been applied in the treatment of human malignancies. Due to the limited availability of interferon, only small numbers of patients have been treated so far. According to the literature a total of 106 patients with malignant lymphomas have been treated, including five patients with Hodgkin's disease, 65 patients with multiple myeloma, nine patients with acute lymphatic leukemia, 14 patients with chronic lymphatic leukemia, nine patients with nodular poorly differentiated lymphocytic non-Hodgkin lymphoma, and four patients with diffuse histiocytic non-Hodgkin lymphoma. Eight of the 106 patients achieved complete remission and 56 showed partial regression [1, 2, 4, 5, 7, 8, 12–15].

A relatively good response was observed with non-Hodgkin lymphoma of favorable prognosis, with 15 partial regressions and two complete remissions out of 23 patients. These results encouraged us to study the effect of fibroblast interferon (Hu-IFN-beta) in patients with non-Hodgkin lymphoma of low-grade malignancy. We describe our results obtained with ten patients. Preliminary results have been reported recently [6].

* This study was supported by the Bundesministerium für Technologie und Raumfahrt, Bonn, FRG

B. Materials and Methods

Partially purified human fibroblast interferon was produced by Dr. Rentschler GmbH und Co., D 7958 Laupheim, Federal Republic of Germany. It was supplied in lyophilized form at a specific activity of 2.5×10^6 IU/mg protein. Freshly dissolved material was applied by intravenous infusion over a period of 30–60 min.

Between October 1980 and August 1981 ten patients entered the study. Their diagnoses were designated according to the Kiel classification [11]. All patients were in advanced stages of their disease, with cytostatic and radiation pretreatment. Such treatment was omitted for at least 1 month prior to interferon application.

C. Treatment Evaluation

Physical examinations took place at weekly intervals; spleen size and lymph node index were recorded. The development of the disease was documented by chest roentgenograms, CT scans, and bone marrow histology. Response was evaluated according to the criteria suggested by the International Union Against Cancer [7].

D. Dosage Schedule

Patients received induction therapy with 4.5×10^6 IU i.v. daily for 4 weeks and thereafter 9×10^6 IU i.v. daily for 2 weeks. After this dose increment consolidation therapy was started, with 4.5×10^6 IU i.v. 3

	n	Result after						
		Induction and increment				Consolidation		
		CR	PR	SD	PD	CR	PR	PD
CLL	1	–	–	–	1	–	–	–
PCLL	1	–	–	–	1	–	–	–
IC	4	–	–	2	2	–	–	2
CC	1	–	–	–	1	–	–	–
CB/CC	3	–	–	2	1	1	1	–
Total	10	–	–	4	6	1	1	2

Table 1. Results of treatment of ten patients with advanced non-Hodgkin lymphoma

CLL, chronic lymphatic lymphoma; PCLL, prolymphocytic leukemia; IC, immunocytoma; CC, centrocytic lymphoma; CB/CC, centroblastic/centrocytic lymphoma; CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease

times per week. Interferon treatment was abandoned when patients fulfilled the criteria for progressive disease [7].

E. Results and Discussion

Ten patients with advanced non-Hodgkin lymphoma of low malignancy were treated with Hu-IFN-beta. One patient had chronic lymphatic leukemia, one patient had prolymphocytic leukemia, four patients had lymphoplasmocytoid immunocytoma, one patient had centrocytic lymphoma, and three had centroblastic/centrocytic lymphoma. All patients were pretreated with chemotherapy and in some cases with irradiation. The outcome of the treatment is summarized in Table 1. During induction and increment therapy six out of ten patients had progressive disease and four out of ten had stable disease; two of the latter developed progressive disease after 1 and 3 months of consolidation therapy, respectively.

In one patient with centroblastic/centrocytic lymphoma, preexisting minimal bone marrow infiltration disappeared, as evidenced by biopsies on both iliac crests. We rated this response as complete remission. In a second patient with centroblastic/centrocytic lymphoma, bone marrow involvement disappeared, while the lymph node enlargement remained constant. We rated this response as partial remission. It is possible that we missed small nodular lymphatic infiltrates in the bone marrow. Therefore these two cases of good response based solely involvement must be viewed with reservation.

All patients experienced the known reversible interferon side effects: fever, chills, malaise, fatigue, and headache. Some patients showed a reversible drop of absolute granulocyte counts.

These results, namely the achievement of only one partial remission and one questionable complete remission out of ten patients are rather disappointing, particularly when compared with the promising data of Merigan et al. [13] and Gutterman et al. [7]. The reasons for these discrepancies are unknown. The differences may in part be due to the use of IFN-beta instead of IFN-alpha [3]. The poor response of the patients described in this study cannot be explained by a lack of stimulation of natural cytotoxicity or by the production of antibodies to interferon. All patients responded with a significant increase of NK-cell activity with peak values 1–7 days after interferon administration and a subsequent drop during prolonged interferon application. No patient developed antibodies against interferon.

We can conclude that IFN-beta certainly does not cause rapid effective tumor regression. It may exert a moderate response or a stabilization of the disease.

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Results of LSA_2-L_2 Therapy in Children with High-Risk Acute Lymphoblastic Leukemia and Non-Hodgkin's Lymphoma *

F. Zintl, J. Herman, D. Katenkamp, H. Malke, and W. Plenert

In the early seventies, overall survival in patients with childhood non-Hodgkin's lymphomas (NHL) was less than 30% at 5 years. The majority of long-term survivors were patients with very limited disease. Subsequently a dramatic improvement in the end results of therapy for children with NHL has been reported. The best overall disease-free survival curves were reported by Wollner and her colleagues using the LSA_2L_2 protocol [4]. In 1978 we adopted this protocol for treatment of childhood NHL in our group. Independently of the stage of disease all children were treated in the same way. At the same time a study was started to evaluate the efficacy of the LSA_2L_2 protocol only for patients with high-risk acute lymphoblastic leukemia.

A. Materials and Methods

I. Non-Hodgkin's Lymphoma

From January 1978 to December 1981, a total of 44 consecutive previously untreated children with NHL were treated with the LSA_2L_2 protocol [4]. No patients were excluded from this therapy.

1. Staging

Prior to the initiation of therapy the stage of disease was assigned according to the Wollner staging system [4]. Retrospectively the patients were restaged according to the staging system used by Murphy et al. [3]. The primary location of tumor and the stage for the 44 children on study are shown in Table 1. Stage IV NHL was defined as less than 25% replacement of the marrow cells by blast cells. ALL was defined by complete or at least more than 25% replacement of the marrow with blasts.

2. Histology

Histology confirmation was obtained in all cases except one. In the 44th patient the diagnosis was established by a typical mediastinal presentation. Cases were classified according to the Kiel classification [1].

3. Treatment

Children with high-risk ALL and NHL all received the same cytostatic therapy ac-

Table 1. Stage and location of primary tumor

Primary sites	No. of pts	Stage I	II	III	IV
Intra-abdominal	16	–	7	9	–
Mediastinal	16	–	–	14	2
Peripheral-nodal	4	1	1	1	1
Naso-pharyngeal	4	1	3	–	–
Extranodal	4	1	1	2	–
Total	44	3	12	26	3

* A Report from the Working Group for Pediatric Hematology and Oncology of the GDR

Table 2. ALL therapy study LSA_2L_2: patient characteristics and results

	n	%	CCR (after 42 months)
ALL patients	82	100	0.389±0.06
Sex:			
Boys	49	60	0.427±0.08
Girls	33	40	0.325±0.09
Age:			
2 – 10 yrs	49	60	0.440±0.08
<2 and >10 yrs	33	40	0.329±0.09
Thymic mass:			
Negative	55	67	0.394±0.08
Positive	27	33	0.370±0.09
WBC:			
$<50\times10^3/mm^3$	29	35	0.402±0.10
$>50\times10^3/mm^3$	53	65	0.375±0.07
CNS involvement:			
Negative	74	90	0.417±0.06
Positive	8	10	–
E-Rosettes:			
Negative	30	57	0.377±0.10
Positive	23	43	0.413±0.10
Acid phosphatase:			
Negative	57	71	0.374±0.07
Positive	23	29	0.432±0.10

cording to the LSA_2L_2 protocol [4]. Laparotomy was done in all NHL children with their primaries in the abdomen. In eight children the intra-abdominal tumor had been completely resected primarily. Eight children had presented with nonresectable tumor. Thirty-five of 44 children received radiation therapy to the involved field. The dose range varied from 11 to 45 Gy. In cases of widespread abdominal disease the whole abdomen was treated to 20 Gy, and second-look laparotomy in the 4th to 5th week of the induction phase was performed. Thirty-five children (stages I–IV) achieving a complete remission received prophylactic treatment of the CNS (10 MTX only, 14 cranial irradiation with 18 Gy and MTX, 11 radiogold intrathecal and MTX).

II. High-Risk Acute Lymphoblastic Leukemia

Eighty-two consecutive children with high-risk acute lymphoblastic leukemia were entered into the nonrandomized LSA_2L_2 study between January 1978 and April 1981. Patient characteristics are given in Table 2. Children with non-Hodgkin's lymphomas who had 25% or more tumor cells in the bone marrow were included.

The criteria for high-risk factors were defined as:

1. Leukocyte counts of 50,000 per/mm^3 and more.
2. Mediastinal mass.
3. T-ALL (blast cells from spontaneous rosettes wich sheep erythrocytes).
4. Positive acid phosphatase reaction of the blast cells.
5. CNS leukemia at diagnosis.

As CNS prevention therapy all children received combined intrathecal injections of methotrexate and prednisolone during induction and consolidation therapy and periodically throughout the continuation treatment. In addition, 30 children received preventive cranial irradiation (18 Gy) and 34 received intrathecal application of macrocolloidal radiogold (range 1.5–6.5 mCi).

B. Definitions

Complete remission (CR) is defined by the absence of all symptoms and signs of lymphoma. The duration of CR is the time from the end of the induction phase and the first sign of relapse. Children who died during remission are considered to be therapeutic failures and counted as relapses. Calculation of actuarial estimates of remission has been performed by the Life Table Method [2].

C. Results and Conclusions

I. Non-Hodgkin's Lymphoma

Forty of the 44 children could be evaluated for their response to induction therapy. Four patients were still not in remission at the cut-off date (31 December 1981). The complete remission frequency was 35 out of 40 (88%). The overall actuarial estimate of

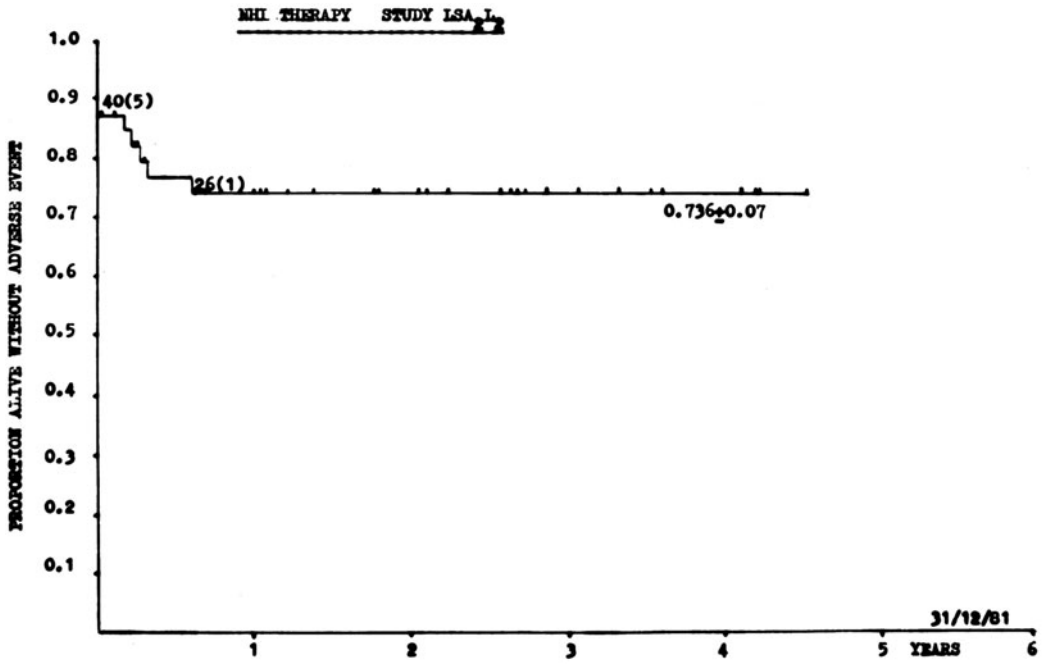

Fig. 1. Actuarial disease-free survival for the total NHL patient population

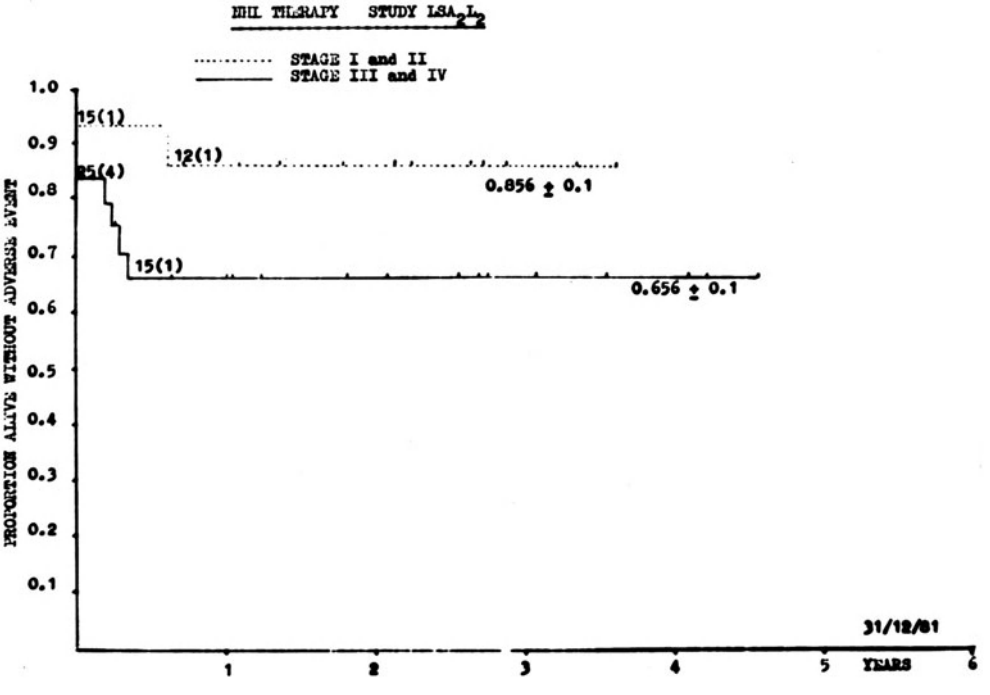

Fig. 2. Actuarial disease-free survival for NHL children with stage I–II disease and stage III–IV disease

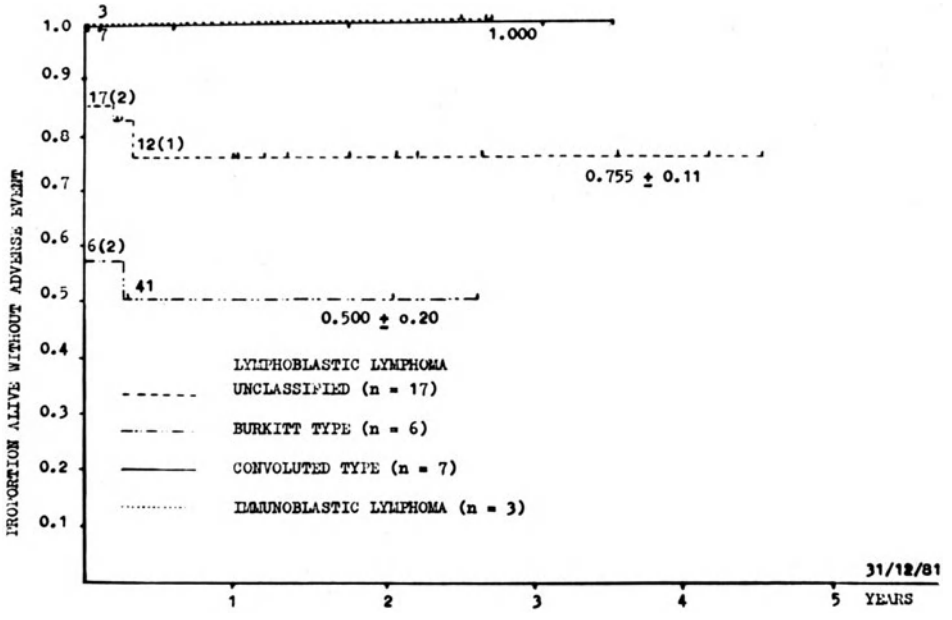

Fig. 3. Actuarial disease-free survival for NHL children with different histology (Kiel classification)

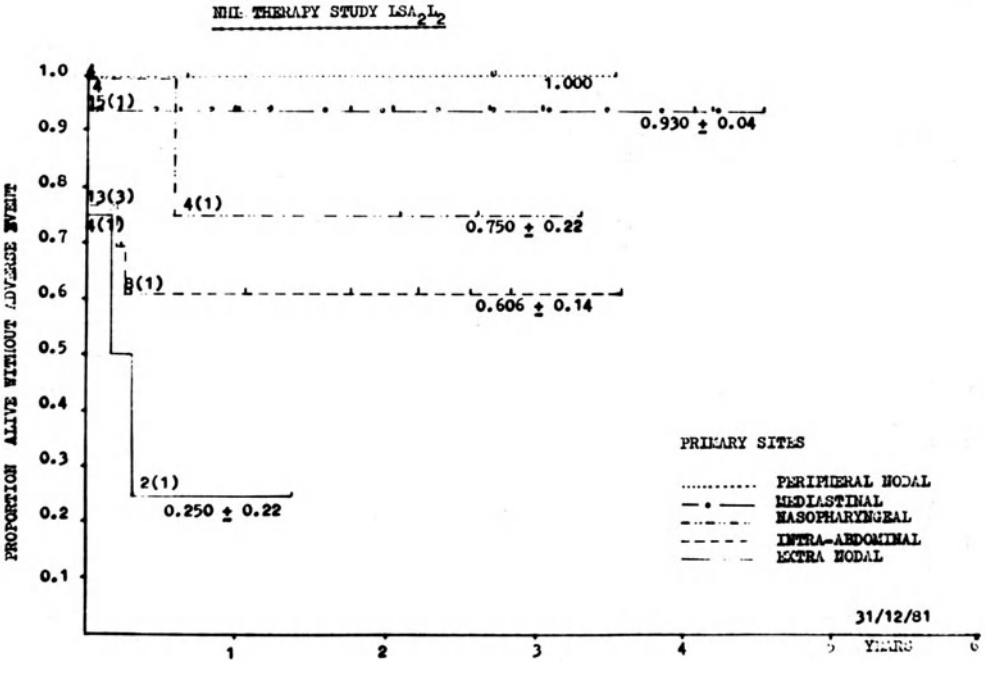

Fig. 4. Actuarial disease-free survival for NHL children with different primary sites of disease

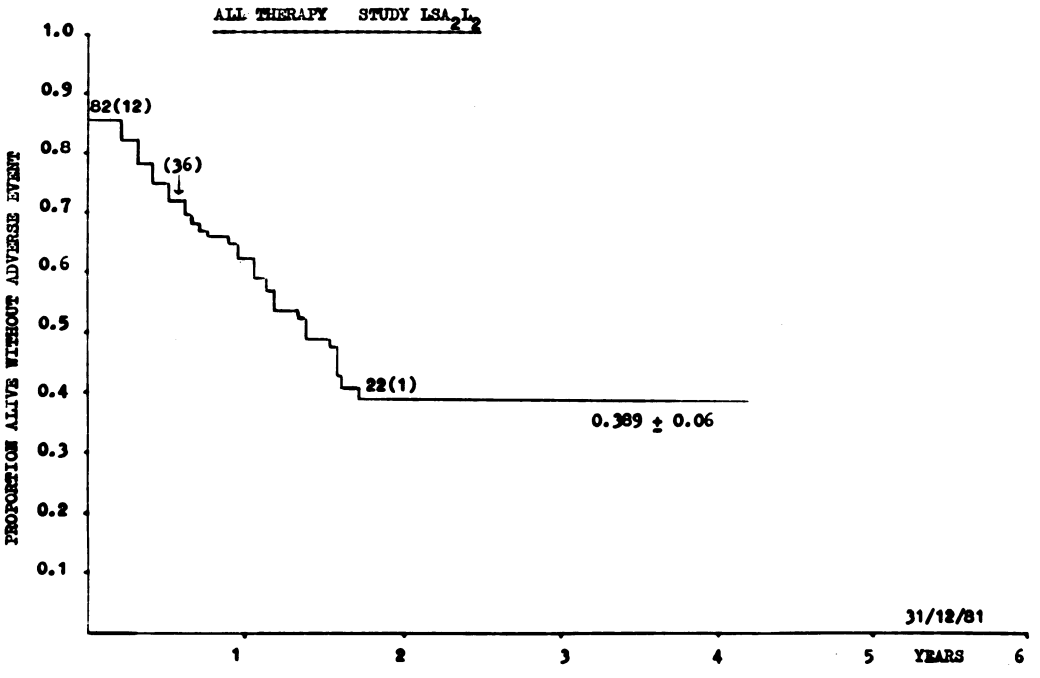

Fig. 5. Actuarial disease-free survival for total high-risk ALL patient population

disease-free survival for complete responders is 73.6% (Fig. 1). Children with stage I and II disease had significantly superior disease-free survival than did children with stages III–IV (Fig. 2). The influence of histological type and the location of the primary tumor on the outcome of treatment is shown in Figs. 3 and 4.

II. High Risk Acute Lymphoblastic Leukemia

The results are summarized in Fig. 5 and Table 2. Seventy of the 82 (85%) attained complete remission after 4 weeks of therapy. Twelve children died during the induction phase. Another three children died during maintenance therapy. The cumulative proportion in continuous complete remission is 0.39 (SD = 0.06) (Fig. 5). ALL relapses [31] occurred during the first 2 years, 12 of them concerning patients with WBC above 100,000/mm^3. Patients with thymic involvement did no worse than those who had no mediastinal mass. Five of eight children with initial CNS involvement died during induction therapy phase or relapsed. CNS relapses occurred only in the radiogold group (8 of 34).

We found the LSA_2L_2 protocol was highly effective for children with lymphoblastic lymphoma (73% relapse-free survival), but inferior (39% relapse-free survival) for patients with high-risk lymphoblastic leukemia.

Patients with stage I or II NHL have similar survival rates irrespective of treatment with the LSA_2L_2 protocol or less-aggressive therapy. A less-aggressive treatment should be used for these children to avoid therapeutic complications and late sequelae.

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Clinical and Epidemiological Observations on Acute Lymphoblastic Leukemia Subtypes at the Sheba Medical Center, Israel

B. Ramot, I. Ben-Bassat, A. Many, G. Kende, Y. Neuman, F. Brok-Simoni, and E. Rosenthal

A. Introduction

Childhood lymphoproliferative disorders have multifactorial etiologies, in which environmental and genetic factors play a major role in determining the age distribution, clinical presentation, and course of the disease. Most of the data, however, are indirect. The gradual appearance of the 3–5 years age peak of common ALL in developed countries and its absence in developing countries and the marked decrease in the incidence of alpha heavy chain disease in Israel are two examples of changes in patterns of disease that can be attributed to environmental factors.

As Israel is a relatively small country with a heterogeneous population, good medical facilities, and central cancer registry, it lends itself for such studies. Furthermore, the Gaza Strip is an ideal region for such studies as marked improvement in socioeconomic status has occurred there during the past decade. Most probably related to these socioeconomic changes is a decrease in the frequency of Burkitt's lymphoma with a concomitant rise in acute leukemia [1].

The present communication is a summary of our experience of acute lymphoblastic leukemia (ALL) subtypes between 1978 and 1981 – a period when cell markers were routinely performed in our center [2].

B. Patient Characteristics and Results

Fifty-two patients will ALL were diagnosed and treated in our center and 49 of them could be classified. Thirty were Arabs from the Gaza Strip and 19 were Jews; 42 were children below the age of 16 and seven were adults. The markers used to classify the patients and the distribution into subtypes are presented in Table 1. In both ethnic groups 30% of the patiens were T-cell ALL and two-thirds had poor risk clinical criteria [3]. The subdivision of the cases according to number of risk factors is given in Table 2.

Table 1. Immunological subtypes of the ALL patients

T-cell		
Definite	13	E^+; mediastinal mass 7/13
Probable	3	E^-; mediastinal mass 3/3; high ADA
	16	
Non-B, Non-T		
Common ALL	10	E^-; sIg^-; $CALLA^+$
Pre-B	4	E^-; sIg^-; cIg^+
Null	5	E^-; sIg^-; cIg^-; $CALLA^-$
Partially characterized	14	E^-; sIg^-
	33	

Table 2. Distribution of patients according to risk factors

Risk factors[a]	0	1	2	3	4
No. of patients	17	9	12	10	1

[a] 1, WBC $> 50 \times 10^9$/liter; 2, Age $< 2 > 10$ years; 3, Mediastinal tumor; 4, T-cell ALL; 5, CNS leukemia

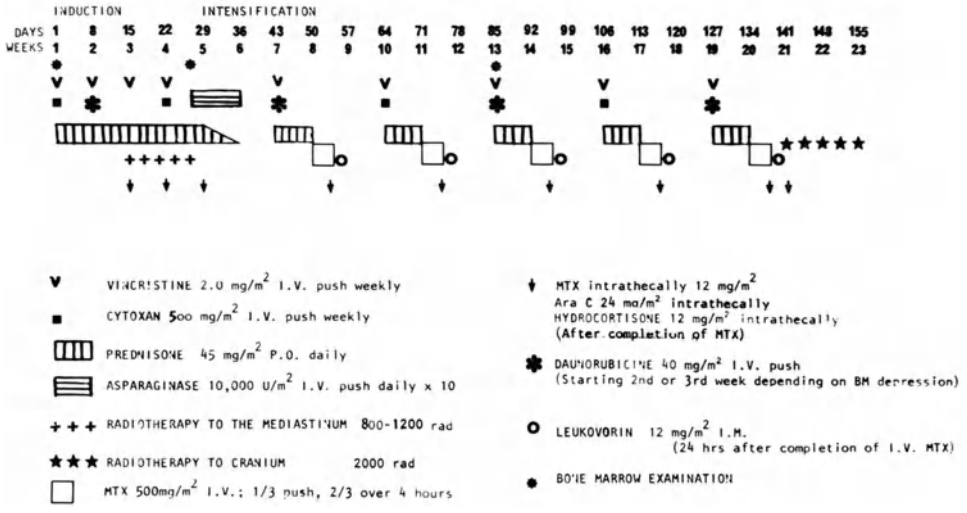

Fig. 1. Treatment protocol for high-risk and other types of ALL and generalized lymphosarcoma

Since 1975, we have used, in addition to the standard immunological markers, adenosine deaminase (ADA) levels as a marker of T-cell ALL [4, 5]. In the present series of patients significant differences in the ADA activity between T-cell ALL and non-B, non-T ALL were confirmed: 44.8 ± 6.3 vs 24.3 ± 3.2 μmol/h per 10^8 cells; $P < 0.0005$. However, the activity of the second purine metabolism enzyme, nucleoside phosphorylase (NP), was not found to differ between the two groups [6].

Our previous observation that Arab children with acute leukemia succumbed within 2 years [3], and the fact that the frequency of acute leukemia among them rose, prompted us to set up a more aggressive protocol for the treatment of poor-risk patients. The details of this protocol are given in Fig. 1. Sixteen children with high-risk leukemia were treated by this protocol – all attained a complete remission, but four relapsed and died of their disease during the 1st year and one died of an unrelated cause. Eleven patients have been in continuous complete remission for 8–55 months. When we analyzed our high-risk patients according to various prognostic factors such as ethnic group, age, initial white cell count, mediastinal tumor, T-cell markers, it was found that out of the seven patients with high ADA four died, while none of the eight patients with ADA levels below the mean of the group (40 μmol/h per 10^8 cells) died of the disease. So it seems from our preliminary data that ADA is an independent risk factor and should be studied in a larger group of high-risk ALL patients [7].

C. Conclusions

Our experience from the Sheba Medical Center would indicate the following: a decrease in the incidence of Burkitt's lymphoma and the increase in ALL in the Gaza Strip Arab children seen in the past decade is most likely due to the socioeconomic development in the Gaza Strip. The present incidence of ALL in this area is about 4 in 100,000 which is similar to that reported from developed countries. Moreover, in this population the minimal estimate of the frequency of T-cell ALL is 30%, which is significantly higher than in Western populations but similar to the observed frequencies in American blacks [8]. However, while in the American blacks the high frequency of T-cell ALL is relative as there is a low incidence of non-T, non-B ALL in the Gaza Strip Arabs, the incidence of ALL is similar to developed countries and so it seems that there is an absolute increase in T-cell ALL.

A similar incidence of T-cell ALL was found in Jews but as we have no country-wide data, no epidemiological conclusions

can be drawn at the present time on the frequency of T-cell ALL in the Jewish population. Such an epidemiological study is ongoing.

As most of our patients are in the high-risk category, we had to introduce an aggressive and intensive chemotherapy protocol. Using this protocol 60% of our patients are in remission – results as good as seen in low-risk cases [3]. ADA activity appears to be an independent risk factor so it would be of great importance to obtain additional data on other populations.

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The Clinical Pharmacology of Cytosine Arabinoside

M. L. Slevin, E. M. Piall, G. W. Aherne, A. Johnston, and T. A. Lister

A. Introduction

There is now little doubt that a proportion of patients with adult acute myelogenous leukaemia will achieve long-term survival and possibly cure with combination chemotherapy. Cytosine arabinoside (araC) is one of the most important drugs used in the treatment of this disease. Despite extensive clinical experience over the past 15 years, the schedule of administration remains controversial. These studies were conducted in an attempt to provide a greater understanding of the effect of both the schedule and route of administration on the clinical pharmacology of araC.

The S phase specificity of araC has led most workers to administer it by continuous intravenous infusion. The potential therapeutic advantages of this schedule have to be balanced against the disadvantages of hospitalisation and the medical and nursing supervision required to maintain continuous intravenous therapy. Some investigators have therefore used subcutaneous bolus injection of araC as a practical alternative to intravenous infusion [1, 12]. The rationale for this was based on the convenience to the patients and the suggestion that following subcutaneous bolus injection araC declined with a half-life of several hours [4]. Methodological problems made this report difficult to assess, and a study was therefore conducted to compare the pharmacokinetics of subcutaneous bolus araC with intravenous bolus and intravenous infusion [13].

Continuous subcutaneous infusion has been successfully used for continuous administration of insulin [10] and desferrioxamine [7], and this route was examined as another alternative to intravenous infusion.

The recent use of massive doses of araC has resulted in a surprisingly high remission rate in patients with acute leukaemia resistant to araC in conventional doses, and may provide a means of overcoming such resistance [3, 8]. The prophylaxis and treatment of central nervous system leukaemia usually requires intrathecal chemotherapy. Patients with neoplastic meningitis may, however, have significant abnormalities of flow which can prevent the even distribution of cytotoxic drugs administered by the lumbar route and may also contribute towards neurotoxicity [5]. Repeated lumbar punctures are often extremely unpleasant for patients. It would clearly be to the advantage of the patient if it were possible to produce prolonged cytotoxic levels of araC in the CSF with the same intravenous therapy used to control the systemic disease. Ho et al. [6] and Canellos et al. [2] have demonstrated that araC crosses the blood-brain barrier during continuous intravenous infusion of conventional doses. It seemed likely that the use of high doses of araC would result in greater CSF araC concentrations than conventional dose infusions, and that these might therefore be useful therapeutically in the treatment of central nervous system leukaemia. The relationship between CSF and plasma levels of araC during high-dose intravenous infusions was therefore studied.

B. Materials and Methods

Patients with acute leukaemia and high-grade non-Hodgkin's lymphoma receiving remission induction or consolidation therapy have been studied. Plasma and CSF araC concentrations were measured using a specific and sensitive radioimmunoassay [9].

C. Results

I. Subcutaneous Bolus araC

The results of this study showed that subcutaneous bolus araC was rapidly absorbed and then declined with a half-life similar to that of intravenous bolus injection. Plasma araC concentrations following a sub-

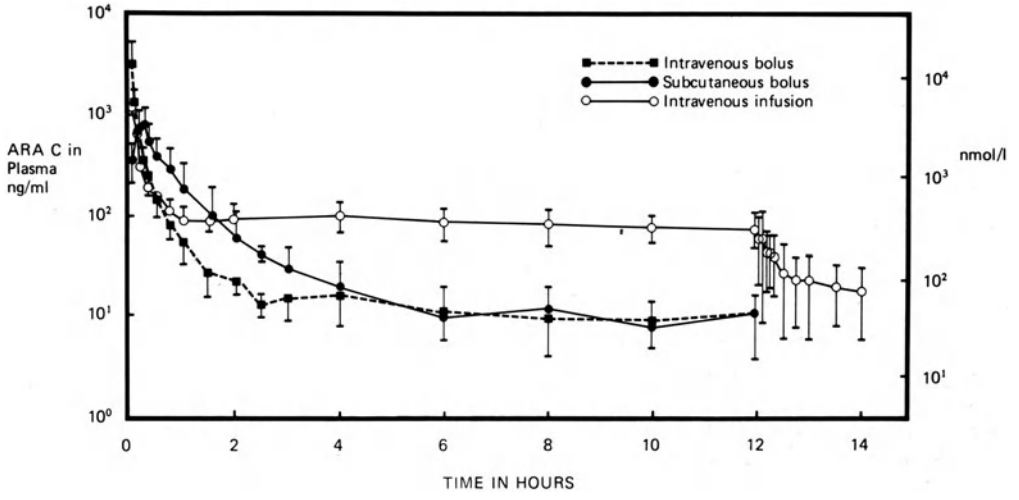

Fig. 1. Mean plasma concentrations (±SD) after intravenous bolus, subcutaneous bolus, and intravenous infusion of araC in five patients

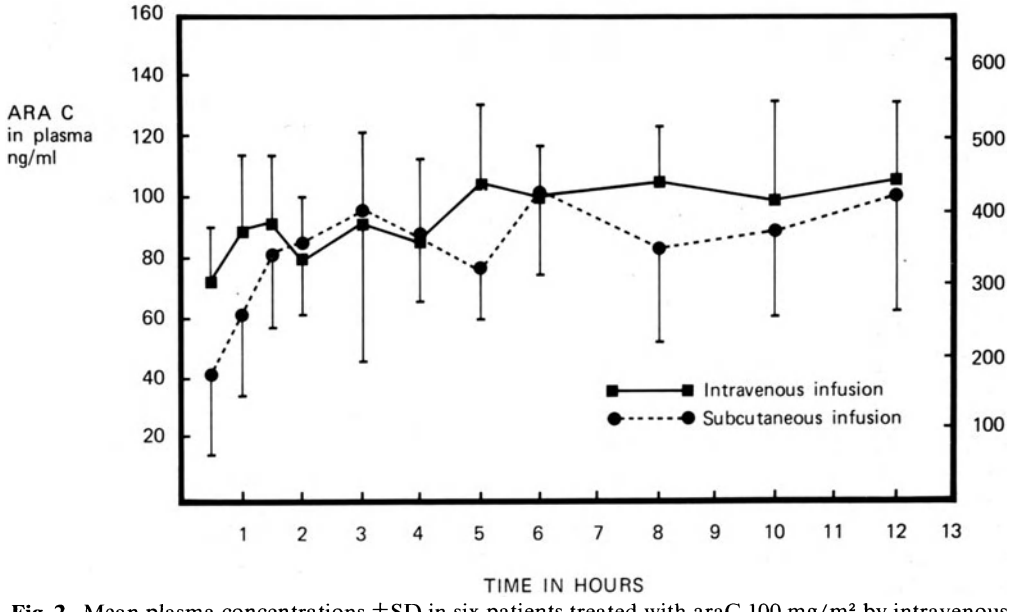

Fig. 2. Mean plasma concentrations ±SD in six patients treated with araC 100 mg/m² by intravenous and subcutaneous infusion over 12 h

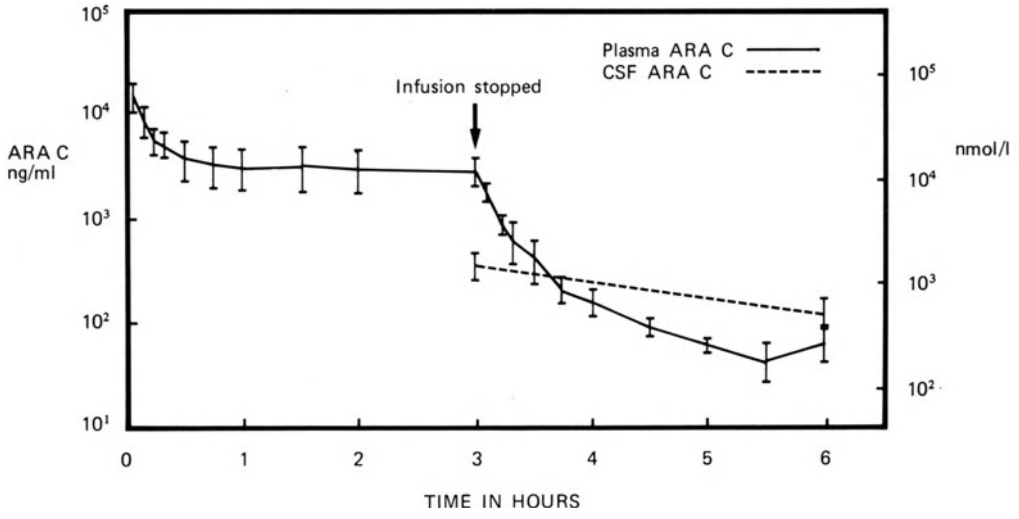

Fig. 3. Mean plasma and CSF levels of araC (±SD) in five patients during an intravenous infusion of 1 g/m² araC over 3 h (one-third of total dose given as a bolus)

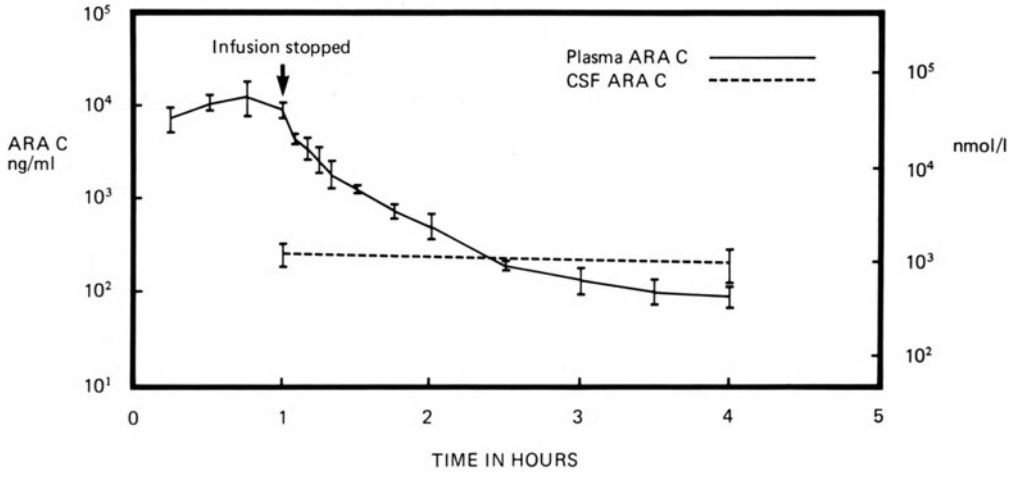

Fig. 4. Mean plasma and CSF levels of araC (±SD) in five patients during an intravenous infusion of 1 g/m² araC over 1 h

cutaneous bolus were greater than those following intravenous bolus only during the first new hours, and within 5 plasma araC concentrations were only 10% of steady state infusion levels (Fig. 1).

II. Subcutaneous Infusion of araC

It was demonstrated that subcutaneous infusion of araC was equivalent to intravenous infusion, and that the time to achieve a plateau, the steady state levels, and the area under the curve were similar for both methods of administration (Fig. 2). There was no local excoriation.

III. High-Dose araC

The relationship between CSF and plasma levels of araC during high-dose infusions was studied. These studies demonstrated that significant concentrations of araC are found in the CSF during therapy with high-dose araC and that levels are similar

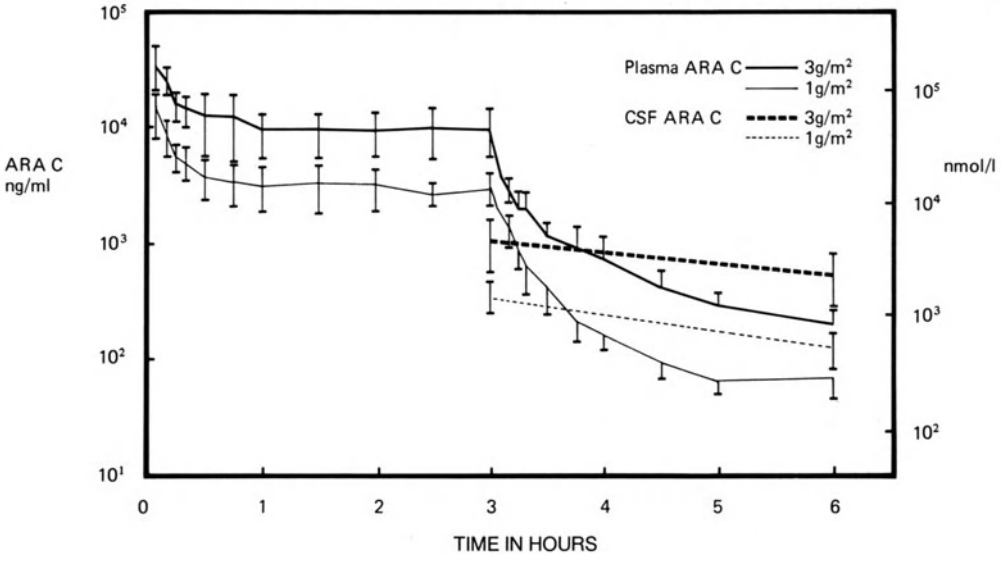

Fig. 5. Mean plasma and CSF concentrations of araC in five patients treated with an intravenous infusion of 1 g/m² and five patients treated with an intravenous infusion of 3 g/m² over 3 h

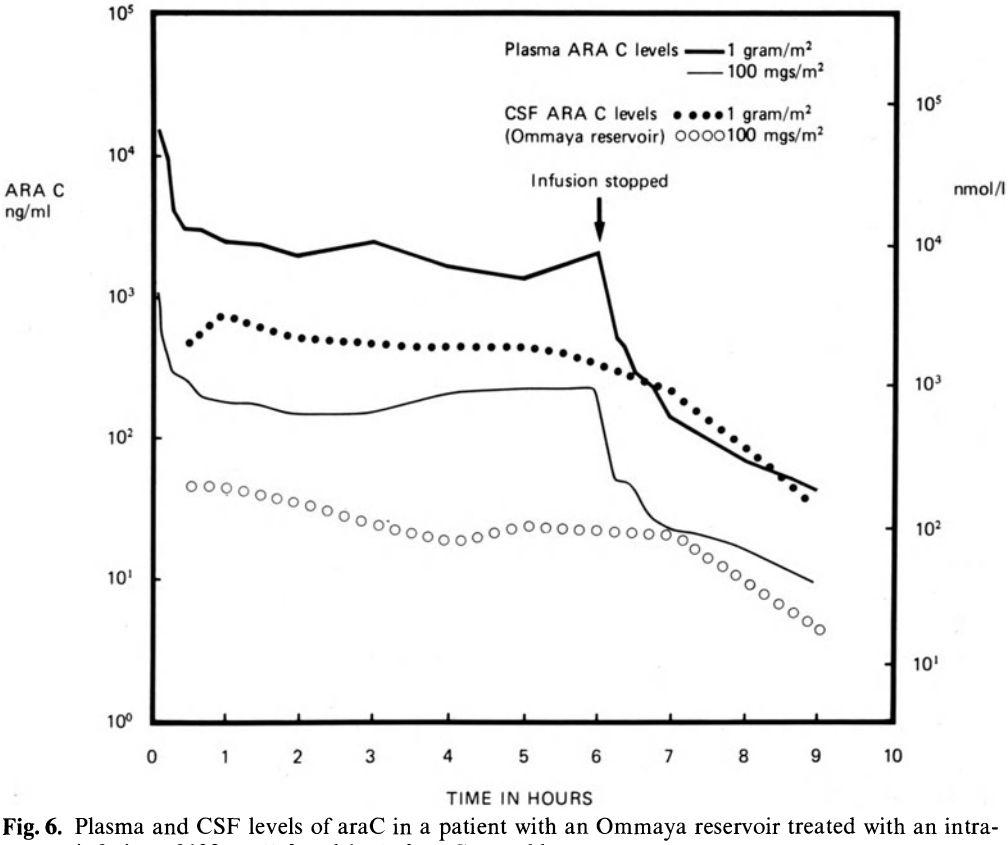

Fig. 6. Plasma and CSF levels of araC in a patient with an Ommaya reservoir treated with an intravenous infusion of 100 mg/m² and 1 g/m² araC over 6 h

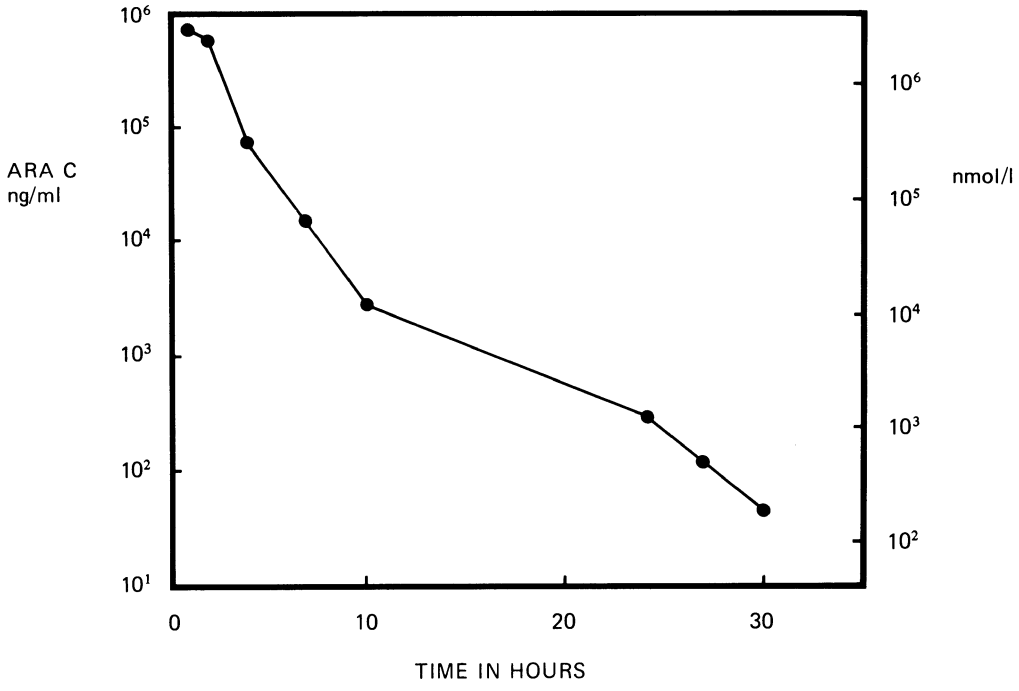

Fig. 7. CSF levels of araC following injection of araC (50 mg/m²) into an Ommaya reservoir

during both 1- and 3-h infusion (Figs. 3, 4). However, the peak plasma concentrations were much greater during the shorter infusions, resulting in a CSF: plasma araC ratio of only 0.03 compared to a ratio of 0.12 during the 3-h infusion. The plasma and CSF araC concentrations during infusions of 3 g/m² were proportionately higher than those found during infusions of 1 g/m² (Fig. 5). Following completion of the infusions, CSF araC levels declined much more slowly than those in plasma with a half-life in excess of 2 h.

The studies conducted in the patient with an Ommaya reservoir (Fig. 6) showed rapid equilibration between plasma and CSF araC, and suggested that araC crossed the blood-brain barrier as effectively during high-dose infusions as at conventional dosage. The CSF concentrations of araC following 50 mg/m² injected into an Ommaya reservoir are shown in Fig. 7. The peak concentrations are several hundred times greater than those found in the CSF during high-dose intravenous therapy, but shortly after 24 h the concentrations have fallen below 100 ng/ml.

D. Discussion

These studies demonstrate that it is not possible to achieve steady state plasma levels of araC comparable to intravenous infusion with the same total dose given by twice or thrice daily subcutaneous bolus injection. Recent data from the CALGB [11], however, showed a significantly longer duration of remission in those patients with acute myelogenous leukaemia who received subcutaneous bolus rather than intravenous bolus maintenance. This suggests that even relatively minor differences between intravenous and subcutaneous bolus araC might significantly affect patient outcome.

Subcutaneous infusion of araC was well tolerated by the patients without local discomfort or excoriation. It is a feasible alternative, and is comparable to intravenous infusion. It allows the patients the advantages of out-patient therapy whilst preserving their venous access.

It is likely that in patients treated with high-dose araC at a dose of 3 g/m² given as a short infusion repeated 12-hourly, CSF

levels >100 ng/ml would be maintained almost continuously. CSF araC concentrations during high-dose therapy are therefore greater than those found in the plasma of patients treated with continuous intravenous infusion at a dose of 200 mg/m² over 24 h. These data suggest that high-dose araC should provide effective therapy for central nervous system leukaemia, and overcome any potential maldistribution of araC in the CSF of patients with leukaemic meningitis.

The central nervous system toxicity associated with high-dose araC cannot be explained in terms of the peak levels achieved in the CSF, as these are considerably lower than those found following intrathecal araC administration. The CNS toxicity is thus most probably related to the prolonged exposure to relatively high CNS concentrations of araC that occur during this therapy.

The most effective schedules of araC administration for both systemic disease and central nervous system leukaemia remain unknown. It is hoped that a greater understanding of the clinical pharmacology will help in the design of studies intended to answer these questions.

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Enhancement of the Cell-Mediated Lysis of Fresh Human Leukemia Cells by Cytostatic Drugs

J. Erhardt, B. Emmerich, H. Theml, G. Riethmüller, and H. W. L. Ziegler-Heitbrock

A. Summary

The in vitro lysis of human leukemia cells by human natural killer cells was enhanced by pretreatment of the leukemia cells with two different cytostatic drugs, which by themselves do not cause lysis of the tumor cells within the time limits of the in vitro assay. Actinomycin D induced this higher susceptibility in four of eight leukemic samples, Cisplatin in three of four samples. Using Actinomycin D pretreatment, no enhanced lysis was seen with lymphocytes and PHA-lymphoblast targets from healthy donors nor with leukemia cell lines. Our results indicate that a larger fraction of leukemia cells than previously detectable can be recognized and destroyed by spontaneous killer cells.

B. Introduction

Attempts to increase the cell-mediated killing of leukemia cells in the past have focused on the activation of the killer cells [2, 4, 7, 8]. Various studies have shown that the killing of cell lines by cytotoxic cells or antibody plus complement can be modulated by cytostatic drug and hormone treatment of the target cells [1, 3, 5, 6]. To our knowledge, fresh human tumor material has not yet been tested with this approach. Therefore we tried to manipulate the susceptibility of the leukemic cells with cytostatic drugs, which by themselves do not kill the tumor cells under the conditions used herein.

C. Material and Methods

Peripheral blood leukemia cells (more than 80% blasts) and mononuclear cells from healthy donors were isolated by density gradient separation and used either directly or stored in liquid nitrogen in the presence of dimethyl sulfoxide. The leukemia cells were incubated with actinomycin D (ActD) or Cisplatin for 2 h, followed by labeling with ^{51}Cr in the presence of the respective drugs for 1½ h. The leukemia targets were then admixed in microtiter plates with serial dilutions of β-interferon-treated mononuclear cells obtained from healthy donors.

The killing exerted by the mononuclear cells was assessed after 6 h of incubation by counting the ^{51}Cr released into the supernatant. Representative values of specific release were taken from the linear portion of the titration curve.

D. Results and Discussion

Leukemia cells from eight patients with various types of acute leukemia were pretreated with ActD and then used as targets in a cytotoxic assay (Table 1). With this approach four of eight leukemias showed an enhanced specific release induced by ActD, including one case where even IFN-activated killers were ineffective (Exp. 11). As a second drug Cisplatin was tested on four different leukemic samples. Lysis by natural killer cells could be enhanced in three of four cases tested, indicating that other drugs besides ActD are able to increase susceptibility of fresh acute leukemia cells for cell-mediated lysis.

Table 1. Effect of actinomycin D and Cisplatin on cell-mediated lysis of leukemia cells

Target cell	Exp. No.	Donor of effector cells	E:T	^{51}Cr-release			^{51}Cr-release		
				– ActD	+ ActD	ActD (μg/ml)	– Cis-platin	+ Cis-platin	Cisplatin (μg/ml)
VG[a] (ALL)	2	ER	40:1	13.0	37.9	0.2			
RI[a] (CML-BC)	8	JO[a]	40:1	13.0	36.0	1.0			
SC[a] (AML)	10	FU[a]	37:1	11.4	23.0	0.2			
STR[a] (ALL)	11	WA	25:1	– 7.7	15.0	0.2			
SU[a] (CML-BC)	12	HB	40:1	20.3	18.5	0.5			
HU[a] (AML)	13	ZI	40:1	12.7	15.5	0.2			
MI[a] (AML)	14	ME[a]	35:1	– 0.5	2.5	0.2			
RD[a] (AML)	15	JO[a]	30:1	– 10.2	– 10.5	0.2			
KR[a] (CML-BC)	C1	ZW[a]	25:1				0.4	8.8	100
VG[a] (ALL)	C2	JO[a]	50:1				2.7	16.4	10
RI[a] (CML-BC)	C3	MA[a]	12:1				3.7	11.4	100
SC[a] (AML)	C1	ZW[a]	25:1				– 1.2	– 7.4	100

The same treatment induced no cell mediated lysis from lymphocytes and PHA-lymphoblast targets in five experiments. SDs were usually less than 5% release. Release from two leukemic cell lines (K562, Molt4) was decreased by ActD treatment (two experiments) E:T = effector to target ratio

[a] N_2 stored before use

In five experiments leukemia cells were not enhanced in their lysability. In three cases no lysis was obtained, which may be explained by a failure of the effector cells to bind to the leukemia targets. In two other instances interferon-activated killers mediated some lysis of leukemia cells, which could not be further increased by pretreatment with ActD. The level of the lysis achieved in these experiments might represent the entire fraction of leukemia cells susceptible to NK cell mediated lysis.

We found the enhancing effect to be restricted to fresh tumor cells, as leukemia cell lines were lysed to a lower degree, while in lymphocyte and lymphoblast targets no lysis was induced when treated with ActD (data not shown). More normal targets, however, will have to be tested to claim tumor specificity of the effect. Nevertheless our findings warrant similar studies employing autologous killer and leukemia cells.

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Secretion of Plasminogen Activators by Human Myeloid Leukemic Cells: Modulation and Therapeutic Correlations

E. L. Wilson, P. Jacobs, and E. B. Dowdle

Plasminogen activator (PA) synthesis and release are inducible cellular functions [1–7] that are subject to modulation by hormones, drugs, and other pharmacological compounds which act at a genetic level. Normal granulocytes synthesize PA [8, 9] and it is known that human cells release, plasminogen activators of two distinct immunochemical types – one similar to urokinase and the other similar to tissue activator [10–13].

In view of these facts we felt that it would be of interest to determine whether acute myeloid leukemic (AML) cells released PA and, if so, whether enzyme type and modulability could usefully be correlated with clinical or prognostic features in an given case. Since the anti-inflammatory steroid, dexamethasone, and the tumor promoter, tetradecanoyl phorbol acetate (TPA), are able to influence PA release by other cells [1, 2, 9, 14–19], these were used in the present experiments.

The results of this study show that leukemic cells secrete both types of PA and that patients with AML whose cells released only tissue plasminogen activator did not respond to combination chemotherapy [20]. Both dexamethasone and TPA could stimulate or inhibit secretion of both forms of enzyme, and PA secretion should prove a sensitive means of monitoring the responses of AML cells to biologically active compounds [21].

A. Methods

Heparinized blood samples were obtained from 18 normal and 69 patients with AML. Cells were isolated by centrifugation on a Ficoll-Hypaque cushion [20] and resuspended in RPMI containing 3% fetal calf serum to give 4×10^6 cells/ml. The appropriate concentration of TPA or dexamethasone was added and the dishes were incubated for 24 h at 37 °C in a humid atmosphere of 5% CO_2 in air. At the end of this period the medium was harvested by centrifugation and replaced with fresh medium containing compounds at the same concentration as before. At the end of the second period of incubation the medium was collected and the samples (harvest fluids) were stored at −80 °C for analysis of PA activity. PA was assayed by measuring the plasminogen-dependent release of soluble radioactive fibrin degradation peptides from insoluble ^{125}I-labeled fibrin-coated Linbro multiwell plates as previously described [22]. Molecular species of plasminogen activators were identified by electrophoretic and immunochemical procedures as previously described [10].

B. Results and Discussion

Fibrinolytic activity released by normal and leukemic cells in culture was invariably and completely plasminogen dependent [20].

I. PA Type and Response to Chemotherapy

Electrophoretic and immunochemical analyses showed that, whereas normal neutrophils invariably released only the urokinase-type of enzyme, cells from 14/69 pa-

Table 1. Correlation between clinical outcome and molecular species of plasminogen activator released by cultured cells of 69 patients with AML

Therapy	Group	Response	Nature of plasminogen activator				Totals
			TA	UK	TA and UK	Unknown	
Combination chemotherapy	A	*Assessment completed*					
		Complete remission	0	21	3	2	26
		No remission	7	5	0	1	13
		(Subtotals)	(7)	(26)	(3)	(3)	(39)
	B	*Died before assessment*	3	10	1	2	16
Palliative/ alternate therapy	C		4	7	1	2	14
Totals			14	43	5	7	69

TA, tissue activator; UK, urokinase

tients with AML secreted tissue activator, cells from 43 patients secreted the urokinase-type enzyme, and cells from five patients secreted a mixture of urokinase and the tissue-type enzymes. Cells isolated from seven patients with AML secreted too little enzyme for the activator to be identified with certainty (Table 1).

The tendency for approximately 20% of AML patients to have cells that released tissue activator was apparent in each of the three major therapeutic subdivisions in Table 1. Thus, blasts from 4/14 patients who received palliative therapy; from 3/16 patients who were treated with standard combination chemotherapy but who died before evaluation could be completed; and from 7/39 patients in whom results of therapy could be assessed released tissue activator. If one considers only the 39 patients in whom therapeutic responses could be evaluated, a satisfactory remission was induced in 83% (24/29) of patients whose cells released the urokinase-type enzyme. In contradistinction, none of the seven patients whose cells released tissue activator alone entered remission. In this limited series, therefore, there was a significant correlation ($\chi^2 = 17.8$; $P < 0.001$) between the release of tissue activator alone and a poor response to the cytotoxic regimen that was used [20].

II. Effects of Dexamethasone and TPA on PA Release

In 35/45 cases, 10^{-7} *M* dexamethasone inhibited PA secretion by at least 25%. Pronounced inhibition (greater than 75%) was observed in 26/45 cases. In 6/45 cases the steroid stimulated enzyme production (greater than 140% of control), and in 4/45 cases no effect on enzyme secretion was observed. The fact than the rate of PA release by cells from 41/45 patients with AML was modulated by 10^{-7} *M* dexamethasone implies that most AML cells possessed receptors for this steroid [21].

Dexamethasone has generally been observed to inhibit PA synthesis by cells cultured in vitro [1, 2, 9, 18, 24], and Roblin [19] has recently suggested that synthesis of the urokinase-type enzyme is suppressed by dexamethasone whereas cellular release of tissue PA is resistant to regulation by glucocorticoids. Our observations with leukemic cells show that release of both types of PA were susceptible to either stimulation or inhibition by dexamethasone [21]. The cell type rather than the enzyme species may therefore be the determinant of dexamethasone responsiveness and, unlike other cell types studied, certain AML cells show stimulation of PA secretion in response to this glucocorticoid.

The effects of TPA on the secretion of PA by AML cells varied considerably. When added at 1 ng/ml the compound caused profound inhibition (greater than 75%) of enzyme release in 20/41 cases and stimulated in 8/41 cases. As found with dexamethasone both species of PA could be inhibited or stimulated by TPA. The effects of both TPA and dexamethasone were inhibitable by actinomycin D and hence required the transcription of new mRNA [21].

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Bone Marrow Transplantation in West Germany in Patients with Leukemia *

K. Wilms, K. Schüch, C. Müller, H. Link, P. Meyer, P. Ostendorf, H. D. Waller, P. Wernet, D. Niethammer, H. Dopfer, W. Schneider, F. Schunter, G. Breitling, W. Frommhold, G. Seeger, A. C. Voss, and H. Rodt

A. Introduction

Bone marrow transplantations (BMT) are being performed in West Germany, the United States, and other European countries with increasing frequency. The first allogeneic BMT in Germany was done in Munich 1975 in a patient with severe aplastic anemia. Since then 128 BMTs have been registered by the German Group for BMT, which consists of four active transplantation centers: Essen, Munich, Tübingen, and Ulm. All these transplantation units are not independent institutions, but are integrated into medical or pediatric departments of university hospitals. Thus all groups treat patients with hematological neoplasias or bone marrow failure in the conventional way as well.

The German Group for BMT organizes regular plenary meetings, stimulates common study protocols, and organizes the assignment of patients from one center to another according to actual capacity. We present the Tübingen experience here, with discussion of the clinical management, results, and experimental approaches.

B. Patients and Methods

Thirty-three BMTs were performed in Tübingen between August 1976 and May 1982. Seven patients suffered from severe aplastic anemia, 25 patients were transplanted for acute leukemia, and one patient was transplanted for chronic myelocytic leukemia in the chronic phase. The first three leukemia patients transplanted in Tübingen in 1976/1977 were in the end stage of their disease and resistant to conventional chemotherapy. All patients died in the early posttransplant period from persisting leukemia or leukemia-related complications. In October 1979 we decided, as the first group in Germany, to perform BMT in patients with leukemia only in remission. This decision was based on the Seattle experience with this procedure [5, 6]. Since that time we have transplanted 22 leukemic patients in remission. Diagnoses and remission status are shown in Table 1.

The age range of the patients was between 3 and 35 years. The donors were identical twins in two cases; the others were siblings with HLA-A, B, C, D and D_R identity, with one exception, a positive MLC. In eight cases there was an ABO incompatibility, requiring a plasma exchange in one case with a very high isoagglutinin titer; in the other cases buffy coat preparations were grafted. All patients were

Table 1. Bone marrow transplantation in patients with leukemia in remission ($n=22$). Diagnoses and remission status

	I.	II.	III.	remission
AML	6	6	1	
ALL		5	2	
AUL	1			
CML	1 (chronic phase)			

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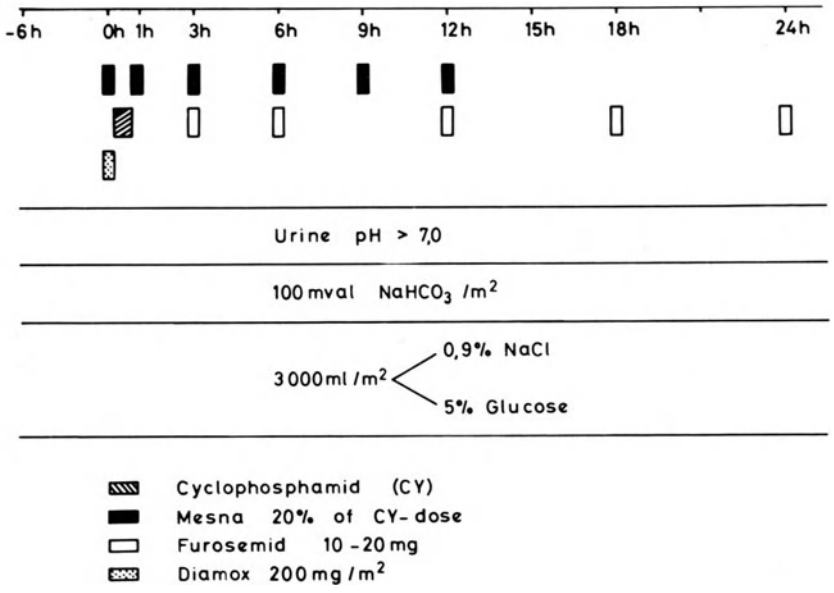

Fig. 1. Protocol for uroprotection with Mesna to prevent cyclophosphamide-induced cystitis

treated under gnotobiotic conditions in Laminar airflow units and received Bactrim prophylaxis for pneumocystis. The conditioning regimen consisted of 60 mg/kg cyclophosphamide on days −5 and −4. Under strict uroprotection with Mesna, according to the protocol outlined in Fig. 1, with shortened application intervals [1] no hematuria occurred.

Total body irradiation was performed on day 0 before marrow infusion, with a 10-MeV linear accelerator at a dose rate of 0.07 Gy/min up to a midline dose of 10 Gy. The lung dose was limited to 8 Gy by satellite technique. Patient No. 16 received a fractionated irradiation with 7×2 Gy on consecutive days. The bone marrow aspiration was performed according to the technique of Thomas and Storb [4]. In our first marrow transplantation we were impressed by striking megaloblastic abnormalities in the newly engrafted marrow, independent of methotrexate. We found severely decreased levels of folic acid in the early posttransplant phase in spite of regular oral substitution, presumably due to diminished absorption caused by the radiation-induced damage to the intestine in the light of the low storage capacity for this vitamin. In view of this experience we give a consequent intravenous substitution of folic acid as a part of our hyperalimentation protocol.

GvHD Prophylaxis by Preincubation with AHTCG

In addition to this general procedure we have investigated in Tübingen, in close cooperation with the Munich group of Drs. Thierfelder and Rodt, the prophylaxis of graft-versus-host-disease (GvHD) by preincubation of the aspirated marrow suspension with rabbit anti-human-T-cell-globulin (AHTCG) for depletion of GvH reactive cells before the marrow infusion. The experimental data and the theoretical background have been published by the Munich group [3].

Until May 1982 we performed this preincubation in 17 patients. The methotrexate regimen was carried out after the marrow graft in the usual way.

C. Results

The patient characteristics and clinical outcome of 22 leukemic patients transplanted in remission can be seen from Table 2. All patients had a documented take. There were no serious complications in the early

Table 2. Clinical data, course, and present status of 22 leukemia patients transplanted in remission

Pat. No.	Age (years)/ sex	Diagnosis remission	Blood group of patient/ donor	Marrow incubation with ATCG +/–	GvH Acute/chronic Grade		Complications	Cause of death	Survival in days from 31.5.82
7	11 M	ALL 2nd	A/A	–	twins		MTX leukencephalopathia		961
8	6 M	AML 2nd	0/0	+	I/II°		Relapse day 691 testes		919
10	19 F	AUL 1st	0/0	+			Interstitial pneumonia	Interstitial pneumonia	65 †
11	25 F	AML 1st	B/0	+	I°	II°	Bronchopneumonia, herpes zoster gen.	Herpes zoster pneumonia cGvH	213 †
12	27 F	AML 1st	A/A	+	I/II°	II/III°	Obstruct. ventil. disorder, bronchopneumonia, herpes simplex stomatitis	CMV, pneumonia cGvH	420 †
14	12 F	AML 2nd	A/A	+			Candida pneumonia, zoster segm., pericardial effusion		592
16	9 M	ALL 3rd	A/0	+	I°	II°	Pulmonary aspergillus, herpes zoster segm.	Lung bleeding	312 †
17	16 M	AML 1st	A/B	+			Interstitial pneumonia	Interstitial pneumonia	33 †
18	3 M	ALL 2nd	A/0	+	I°				392
19	29 M	AML 1st	A/A	–		III°	CMV hepatitis		377
20	14 F	ALL 2nd	A/A	–	twins				338
21	35 M	AML 2nd	A/A	+			Relapse day 112, pulmonary aspergillus	Cerebral hemorraghia	123 †
22	10 M	ALL 2nd	0/0	+	I°				268
23	34 M	AML 2nd	0/A	–			Interstitial pneumonia	Interstitial pneumonia	86 †
24	17 M	AML 2nd	0/B	+					205
25	17 M	ALL 3rd	0/A	–	III°	III°	cGvH and liver insufficiency	Sepsis cGvH	78 †
26	21 M	AML 1st	A/0	+	III°	II°	cGvH and liver insufficiency, herpes simplex stomatitis		93 †
27	26 F	AML 1st	A/0	+					135
28	15 F	CML chronic phase	0/0	+					114
29	24 F	AML 3rd	B/B		I°	I°	Hepatitis (?)		86
30	20 F	AML 2nd	A/A				Hepatitis B, herpes simplex stomatitis		44
32	25 F	ALL 2nd	0/0						9

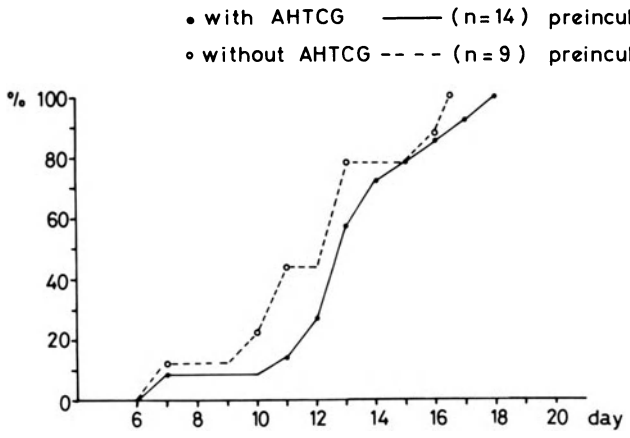

Fig. 2. Time interval from transplantation to documentation of bone marrow take

posttransplant period or deaths related to the transplant procedure. No interference of the AHTCG preincubation with the bone marrow take was observed (Fig. 2). In the later posttransplant period nine patients have died so far. The actuarial survival rate after 1 year is 55%. Major complications were infections, chronic GvHD, and a combination of both. The causes leading to death in these nine patients can be seen in Table 3. Only two out of 21 patients had a leukemic relapse more than 50 days posttransplant. Patient No. 8 developed a testicular relapse on day 691. In patient No. 21, who was transplanted for AML in second remission after rather short duration of the first remission, rapidly progressing bone marrow failure was the first evidence for a relapse, which was diagnosed on day 112. The patient died in overt leukemia from cerebral hemorrhage.

Acute GvHD was not a major clinical problem. We assume that this was due to the AHTCG preincubation: Out of the 16 patients at risk, only one, who was grafted with preincubated marrow cells, had a clinical relevant acute GvHD (grade 3). A transient skin rash with later slight increase in liver enzymes and without intestinal symptoms was observed in 8 out of the 16 patients at risk.

Table 3. Causes of death after allogeneic bone marrow transplantation in patients with leukemia in remission (9/22 patients)

	n
Chron. GvHD with infectious complications	5
Interstitial pneumonia	2
Viral encephalitis	1
Leukemic relapse	1

In contrast major problems were caused in our series by chronic GvHD. We observed this syndrome in six patients, in five after engraftment of preincubation marrow. We were able to favorably influence the clinical symptoms by prednisolone/azathioprine treatment, but five patients with chronic GvHD died from infectious complications due to the severe immunodeficiency caused by this disease. Of particular interest was an obstructive ventilation disorder which occurred in two patients after the onset of chronic GvHD. Treatment with corticosteroids, antibiotics, and adequate broncholytic measures did not prevent progression. Both patients developed recurrent pneumothorax as a later complication. Progressive respiratory failure and subsequent overwhelming infections were fatal in both cases. Interstitial pneumonia as typical complication in the later posttransplant period occurred in five patients, in only two cases without association of chronic GvHD. This was due to cytomegalovirus and varicella-zoster virus respectively and of an unidentified etiology in three cases.

We conclude from our experience that preincubation with the Munich AHTCG reduces morbidity and mortality of acute GvHD but does not prevent chronic GvHD. So in our opinion it is a logical consequence to combine this procedure

with other methods to control immune reactions or induce tolerance. In this direction we have initiated a protocol to combine AHTCG preincubation with cyclosporin A treatment.

In the context of these objectives of improving the control of the potentially life-threatening GvH reaction, improvement of the diagnostic tools and a better pathophysiological understanding of this condition are important. Since 2 years Müller in our group has been supplementing the histological estimation carried out by F. R. Krüger (Cologne) by immunohistological analysis with monoclonal antibodies [2]. It is too early to draw definite conclusions, but the preliminary results can be summarized insofar that in GvH reactions lymphoid infiltrates consist of mature T cells, that there is a focal or generalized appearance of Ia-like antigens on keratinocytes, and that there is a partial reduction of Langerhans cells.

D. Conclusion

The impact of BMT on the treatment of acute leukemias and chronic myelocytic leukemia cannot yet be estimated definitely. We think that with the increased rate of complete remissions in adult AML by the improvement of induction regimens in the past years and the limited results in conventional maintenance therapy for remaining in continuous remission, BMT in first remission should be considered a treatment strategy with curative potential in patients with histocompatible sibling donors under the age of 35 years. In a common protocol with the Ulm group we treat our AML patients according to a regimen which integrates BMT for those patients with an available donor (Fig. 3). This protocol has also been taken up by other non transplanting members of the South German Hemoblastosis Group. In ALL we recommend transplantation in adults still in second remission in addition to high-risk patients.

The indication for BMT in children has been widely discussed in the German BFM Study Group, with the conclusion that transplantation in AML in first remission is not generally acceptable. Children with ALL, besides rare cases of B-ALL, should be transplanted in second remission. In CML in adults and children risk factors must be evaluated for an individual decision.

The four German centers have an annual transplantation capacity now of about 10–15 each. Two other groups are in preparation. The future will show if this capacity is sufficient when, we hope, favorable long-term results are produced and the potential complications of GvHD and interstitial pneumonia can be prevented or treated effectively.

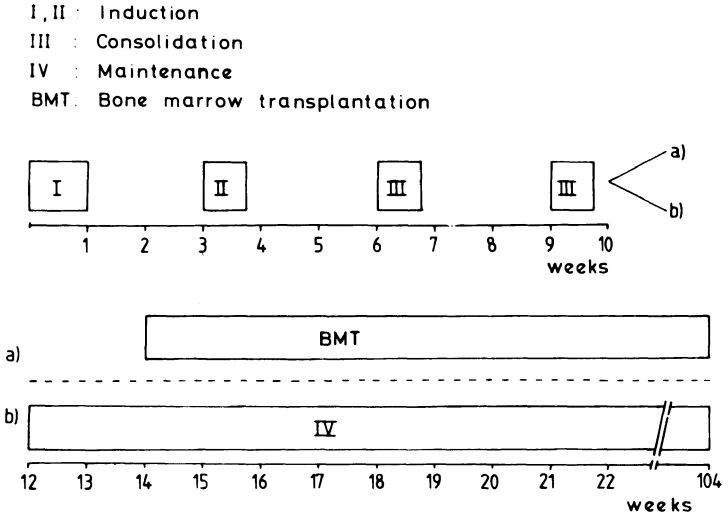

Fig. 3. Schematic outline of the present protocol for adult patients with acute nonlymphoblastic leukemia achieving complete remission

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Cyclosporin A Following Matched and Mismatched Family Allogeneic Bone Marrow Transplants

R. L. Powles, G. R. Morgenstern, M. Leigh, J. Filshie, J. Platt, and H. Lumley

A. Introduction

Following the use of Cyclosporin A (CSA) in patients with established acute graft versus host disease (GVHD) [1] we initiated a study of CSA to prevent GVHD, because it seemed probable that it would be more effective in this role than for reversing the established process. We initially reported the results of 20 patients who had received matched allogeneic sibling bone marrow transplants and prophylactic CSA for longer than 3 months, only one of whom died of GVHD [2].

B. Present Study

Eighty-four patients with acute leukaemia in remission or early relapse or chronic granulocytic leukaemia in the chronic phase have been included in this study, 60 of whom have had follow-up for at least 1 year. All received a matched transplant from an HLA/MLC compatible sibling after conditioning with cyclophosphamide (1.8 g/m^2 intravenously on two consecutive days) (68 patients) or melphalan (85–100mg/mg^2 i.v. as a single dose) (nine patients) followed 36 h later by total body irradiation (TBI) of 10 Gy from a single cobalt^{60} source at approximately 0.03 Gy/min. Seven patients were conditioned with melphalan alone (180–250 mg/m^2 i.v. as a single dose). Marrow was infused from the sibling donor 24 h after the irradiation or 2–4 days after melphalan alone. The patients were nursed in protective isolation (for approximately 4 weeks until reconstitution) and given therapeutic antibiotics, platelets and granulocyte transfusions as required. The CSA was given intramuscularly 25 mg/kg per day (in two 12 hourly doses) starting 24 h before the infusion of the marrow and continuing for 5 days. From the 6th day, i.e. 4 days after bone marrow transplantation, the drug was administered orally and continued for 6 months at a dose of 12.5 mg/kg per day in two divided doses. This dose was reduced by 50% if the blood urea rose above 20 mmol/litre or there was other unacceptable toxicity and was further reduced if the urea continued to rise. Part way through the study the protocol was changed and patients received oral CSA at a dose of 12.5 or 37.5 mg/kg per day for the first 5 days instead of the intramuscular preparation. The intramuscular route was reinstarted after 23 patients.

C. Results

A retrospective analysis of 26 patients receiving methotrexate as prophylaxis against GVHD showed that 46% of the patients developed acute GVHD and 27% of the patients died of the problem. Of the 84 patients receiving CSA, only three (4%) have died of acute GVHD. However, there was a significant incidence (37%) of biopsy proven acute skin GVHD occurring at the time after grafting when we previously saw fatal GVHD. However, all but three of these patients had resolution of their skin disorder, some of whom received treatment with high-dose methylprednisolone. We do not now feel justified in conducting a controlled trial of CSA versus no treatment to

establish the exact role of this drug in preventing GVHD because the incidence of fatal acute GVHD in our series is so low. However, the Seattle Transplant Group has at present a study comparing CSA with methotrexate following bone marrow transplantation. Although such studies are critical for deciding the best treatment for these patients they alone cannot define the exact role of CSA in preventing GVHD in man, because there has not been a study of methotrexate versus no treatment in man.

That there is an effect of CSA on acute GVHD can be established indirectly from our four patients who, after bone marrow transplants and subsequent relapses, were given high-dose melphalan (180–200 mg/m^2 as a single infusion) followed by a marrow transplant from the same donor as the original transplant. None of these patients had developed acute GVHD when given CSA during the initial period after their first transplant. They were not given the drug after the second transplant and two developed acute GVHD (days 20, 44), one of whom died.

The effect of stopping CSA after 6 months also indicates the influence of the drug on preventing acute GVHD. In our previous methotrexate study, where the drug was given for 102 days after transplant, we did not see the development of acute GVHD longer than 52 days after transplantation. Thirty-eight of the 60 patients in the present study have had their CSA stopped approximately 6 months after transplant and these patients have been followed-up for at least a year from transplantation. Twenty-one of these patients subsequently developed acute GVHD, chronic GVHD or a rash that mimicked the syndrome but was not proven histiologically. The timing of these problems occurs as if the patients had received a bone marrow transplant at the time of stopping CSA. Thirteen of these patients were restarted on CSA and seven received azathioprine and prednisolone (some received both treatment regimens). At present 26 of the 38 patients are alive (16 on no treatment) and 12 have died: nine of relapsed leukaemia and three of infection. It is clear that, unlike patients who have received methotrexate after bone marrow transplantation and then developed acute GVHD where we see little clinical benefit from CSA, patients who have previously received prolonged CSA respond well to re-introduction of the CSA.

D. Mismatched Bone Marrow Transplantation

Following the encouraging results of CSA in matched transplants we embarked upon a study using family member donors who had a varying degree of MHC antigenic mismatch.

Thirty-five patients aged 4–45 years with acute leukaemia have been given allogeneic mismatched bone marrow transplants over a period of 3 years. One donor was a two haplotype mismatch; one differed from the recipient at the D locus only because of BD recombination and the remaining 33 were genotypically one haplotype matches, although more than half of these shared some or all antigens on the mismatched haplotype. In all but one instance there was a positive mixed lymphocyte reaction between donor and recipient. Thirty-one patients were conditioned with cyclophosphamide and TBI as for matched transplants, and four were conditioned with high-dose melphalan (240 mg/m^2) only. Patients received intramuscular or oral CSA for the first 5 days from the day before transplantation followed by oral CSA for 6 months as above. Seven of the first eight patients developed a leaky vascular syndrome (see below) and so a new protocol with the addition of methotrexate during the first 6 days after transplant was devised. The rationale for this has been discussed elsewhere [3]. Fifteen patients received this new protocol – no advantage was seen to accrue from it and five patients had failure of graft take and required regrafting. Thereafter patients reverted to the original protocol and received CSA alone (four of whom were conditioned with melphalan without TBI). Preliminary results are that of the 16 patients conditioned with TBI followed by CSA alone, six remain alive, two have died of leukaemia and two of infections and the remaining six have died of the syndrome involving leaky vascular endothelium, usually resulting in fatal pulmonary oedema with or without a concurrent viral infection. Of the 15 patients

conditioned with TBI followed by CSA plus methotrexate, five are alive, one of whom has relapsed. Ten have died, one of leukaemia, one of acute GVHD, one of a failure of graft take followed by infection and the remaining seven with the leaky vascular endothelial problem with or without a concurrent viral infection.

Engraftment using mismatched marrow has been established in three of four patients receiving conditioning, with melphalan alone. The fourth has required regrafting and is too early to assess. GVHD has not been a major problem and this study will continue.

Pulmonary oedema secondary to leaky vascular endothelium has complicated our mismatched bone marrow transplant programme. Approximately 40% of the patients have died of this syndrome and there have been severe fluid balance problems in many of the remaining patients, particularly during the first 30 days after transplant. A similar syndrome occurred in an occasional patient following a matched transplant (less than 10%) and its possible aetiology has been discussed elsewhere [3]. There is an increased incidence of viral infections in our mismatched bone marrow transplant patients, but it is difficult to assess the significance of these and their possible role in the leaky vascular problem. Many more patients will be required in this study to determine the nature of these problems, but we may be able to improve results by empirical methods. The major prognostic factor for success in mismatched bone marrow transplants appears to be age. Of the 20 patients over the age of 20 who were transplanted, only five remain alive; one of these has relapsed and two are within 30 days of transplant and too early to assess. Fifteen patients under the age of 20 have received mismatched transplantations and eight are alive and in remission (40% actuarial plateau), the longest at 2½ years after transplantation.

E. Summary

One hundred and nineteen patients with leukaemia have received CSA as prophylaxis against acute GVHD following sibling MHC matched (84) or family member mismatched bone marrow transplants (35). Four recipients of matched transplants (3%) died of acute GVHD, a marked improvement on our previous results using methotrexate (26 patients; 27% died of GVHD). Thirty-five patients received mismatched transplants, 15 were under the age of 20 years and eight are alive and in remission, the longest survivor being one at 2½ years after transplant.

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Autologous Bone Marrow Transplantation in Acute Leukemia and Non-Hodgkin's Lymphoma: A Phase I Study of 4-Hydroperoxycyclophosphamide (4HC) Incubation of Marrow prior to Cryopreservation *

H. Kaizer, P. Tutschka, R. Stuart, M. Korbling, H. Braine, R. Saral, M. Colvin, and G. Santos

The presence of occult tumor cells in remission marrow is the initial obstacle to the use of autologous bone marrow transplantation in acute leukemia. In vitro treatment of tumor-marrow mixture, with heterologous antiserum and complement, has been shown to be capable of eliminating all clonogenic tumor in a number of animal models, and this approach forms the basis for a number of ongoing clinical trials [1]. An alternative procedure, utilizing in vitro drug treatment of marrow, was first investigated at The Johns Hopkins Oncology Center using 4-hydroperoxycyclophosphamide (4HC). In aqueous solution, this congener of cyclophosphamide (CY) has the same alkylating and cytotoxic effects and the same immunoreactive products as the microsomally activated parent compound.

The initial studies of this approach utilized a model of acute myelogenous leukemia (AML) in the Lewis-Brown Norway (LBN) hydrid rat and involved the inoculation of lethally irradiated rats with marrow tumor cell mixtures which had been treated with various doses of 4HC. These experiments revealed a dose-dependent clearing of tumor cells [2]. Since the LBN-AML model displays an unusual sensitivity to the anti-tumor effects of CY, a model of AML in the Wistar-Furth (WFU) rat, which is not as sensitive to CY as the LBN model, has recently been studied. Despite the relative in vivo insensitivity of the WFU model, similar results have been obtained in treating marrow tumor cell mixtures with varying concentrations of 4HC. Complete elimination of tumor cells has been achieved at concentrations of 60–80 n*M*/ml (18–24 μg/ml) of 4HC. Increasing the concentration of 4HC beyond 80 n*M*/ml resulted in the death of a high fraction of the rats due to marrow failure [3].

We have begun a two-phase study for patients with acute leukemia, either lymphoblastic (ALL) or nonlymphoblastic (ANLL), using 4HC treatment of marrow. The goal of the first phase of this study, which is still ongoing, is to determine the maximal concentration of 4HC that can be used for in vitro treatment and still achieve hematologic recovery. The goal the second phase of the study will be to determine if treatment with 4HC will eliminate all clonogenic tumor.

Thus far, a total of 20 patients with acute leukemia and five patients with non-Hodgkin's lymphoma have been treated on this study as shown in Table 1. This is a dose escalation study. Remission marrow is harvested and aliquoted into a treated and a reserve fraction. The latter is intended for use only if the patient shows no evidence of hematologic recovery after reinfusion of the treated marrow. The pretransplant cytoreductive regimen for ALL and NHL involves CY and total body irradiation. This differs from the regimen used in ANLL which uses CY and busulfan. Both these preparative regimens have been previously described [4]. Although the morbidity of the procedure is significant, only 1 of the 25 patients has had a transplant-related death. All of the other patients have been dis-

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Conc. of 4HC	ALL	ANLL	NHL	% inhibition of CFU-C
40 μg/ml	4	0	1	73
60 μg/ml	2	2	3	85
80 μg/ml	3	3	0	97
100 μg/ml	5	1	1	100

Table 1. Phase I-4HC study patients to July 1, 1982

charged from the hospital in good clinical condition (the average post-transplant stay has been 35 days) and have been able to resume normal activity. Table 1 also shows the degree of inhibition of granulocyte and macrophage colony forming cells (CFU-C) observed as a consequence of 4HC incubation. Despite the high degree of inhibition, essentially no CFU-C detected after incubation at the higher 4HC concentrations, hematologic recovery has been satisfactory in all patients except the one who died of a transplant-related complication (veno-occlusive disease of the liver) too early to evaluate for hematologic recovery. Among the 24 patients evaluable for tumor status, nine remain in remission. Most of those have relatively short observation times, although one ALL and one ANLL patient have been in complete remission for over 15 months. While the disease-free status of those patients contributes to our evaluation of the therapeutic efficacy of 4HC incubation, the ultimate test will be on data collected in the phase II part of the study. This cannot be instituted until the maximal concentration of 4HC is established.

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Serological Inhibition of Graft Versus Host Disease: Recent Results in 28 Patients with Leukemia

H. Rodt, S. Thierfelder, C. Bender-Götze, R. Dopfer, F. J. Haas, G. Janka, H. J. Kolb, H. Link, B. Netzel, D. Niethammer, K. Schüch, and K.Wilms

One major obstacle to the successful application of allogeneic bone marrow transplantation is the occurrence of immunologic complications when donor and recipient are not monozygous twins and express differences in their histocompatibility properties. Even after transplantation of HLA-identical and MLC-negative marrow grafts the occurrence of graft versus host disease (GvHD) cannot be excluded due to genetic differences not detected by present histocompatibility typing techniques. In the past, several experimental approaches have been designed to eliminate the GvH-reactive cell populations in the donor marrow by incubating the graft in vitro with specific antibody preparations whose stem cell toxicity had been absorbed by various tissues, in particular non-T-lymphocytes. Pretreatment of donor spleen marrow with absorbed anti-T cell antisera suppressed GvHD in over 90% of H-2 incompatible semiallogeneic mice preirradiated with 900 R [1]. Also monoclonal Anti-Th-1 antibodies have been effective in preventing GvH reactions in incompatible murine combinations [2]. A suppressing effect of GvHD by incubation with T-cell-specific antibodies could also be shown in a canine model transferring incompatible DLA homozygous marrow to DLA heterozygous lethally irradiated littermates [3]. Since then an anti-T-cell globulin for human cells with comparable serological characteristics has been developed [4]. The present report describes the effect of an incubation treatment with anti-T-cell globulin in 28 cases of bone marrow transplantation in acute leukemias.

The production and serologic characterization of anti-human-T-cell globulin (ATCG) was described elsewhere [5, 6]. ATCG was highly active against T-lymphocytes but did not crossreact with hemopoietic progenitors in the CFU-c test and the diffusion chamber test. Twenty-eight patients with acute leukemia were transplanted between February 1978 and May 1982 with bone marrow of HLA-compatible siblings. Twelve patients were transplanted by members of the Munich Cooperative Group of Bone Marrow Transplantation. These patients received a conditioning regimen including a combined chemotherapy and 1000-rad total body irradiation (TBI) applied by two opposite ^{60}Co sources. In nine cases the chemotherapy included BCNU, cytosine arabinoside, and cyclophosphamide; in three additional cases BCNU was omitted from the protocol. Sixteen patients were transplanted by members of the Tübingen Cooperative Group of Bone Marrow Transplantation, where they received a conditioning treatment with cyclophosphamide and 1000-rad TBI applied by a linear accelerator. In these cases the lungs were shielded to a reduced dose of 800 rad.

The pretransplant status and the treatment protocols before BMT of the described 28 patients are shown in Table 1. Seven of the patients were transplanted after the second relapse, three patients after the first, one patient after the third, and one patient after the fourth relapse. Fifteen patients were transplanted in remission. Five patients had acute lymphoblastic leukemia (ALL). Fourteen had acute myelo-

Table 1. Characteristics of 28 leukemic patients receiving ATCG-incubated marrow grafts

Leukemia status	1st	2nd	3rd	4th[a]	Total[a]
Relapse	3	7	1	1	12
Remission	7	6	2		15
Chronic phase					1
Type of leukemia					
Acute myeloid leukemia (AML)					14
Acute lymphoblastic leukemia (ALL)					10
Chronic myeloid leukemia (CML)					1
Erythroleukemia					1
Acute undifferentiated leukemia (AUL)					2
Treatment before BMT					
BCNU, araC, Cy, TBI-950R[b]					9
AraC, TBI-950R[b]					3
Cy, TBI-1000R[c]					16

[a] Number of patients
[b] Total body irradiation 950 R, performed with $2 \times {}^{60}Co$ sources
[c] Total body irradiation 1000 R performed with a linear accelerator, lungs shielded to 800 R

geneous leukemia (AML). Two other patients suffered from an erythroleukemia and an acute undifferentiated leukemia (AUL), respectively. One patient was transplanted during the chronic phase of chronic myeloid leukemia (CML).

In vitro treatment of the bone marrow was performed as follows: After preparation of bone marrow from the donor, it was first separated from erythrocytes and reduced to a smaller volume, followed by incubation with ATCG. In 11 cases, the marrow was separated using a Hemonetics cell separator. In 15 cases, the marrow was reduced by these techniques to about 300 ml. Two marrow preparations were left unconcentrated. In all cases the marrow was then incubated with ATCG in a final dilution of 1:200 (0.05 mg/ml) for 30 min at 4 °C under gentle agitation. After incubation, the marrow cells were infused without further delay. An average of 6.1 (cell separator), 4.1 (buffy coat), and 3.5 (no separation) $\times 10^8$ nucleated cells/kg body wt. were transferred. Details are shown in Table 2. In all cases the incubated cells were tolerated without severe side effects. Symptoms seen in some of these cases, like frequent pulse or transient fever, have also been described for transplantations without incubation.

Documentation of marrow engraftment and incidence of GvHD after incubation with ATCG are shown in Table 3. In general, engraftment and recovery of bone marrow functions after incubation treatment seemed to be not different from that in other transplantations. Engraftment was documented by bone marrow cellularity and rise of peripheral blood cell counts. Eleven of the 28 patients were sex mismatched, five showed major ABO blood group incompatibility, two were HLA-D different from the donor, and in two others the HLA-D compatibility was unclear. Twenty-six of the 28 patients showed an engraftment between days 12 and 26 post-transplantation, indicated by a rise in the

Table 2. In vitro treatment of bone marrow with ATCG

Concentration of bone marrow	Number of patients	Volume[b]	Nucleated cells[c]		Incubation with ATCG[a]
			Total ($\times 10^9$)	Per kg body wt. ($\times 10^8$)	
Hemonetics cell separator	11	310	25.8	6.1	1 : 200 4 °C, 30 min.
Buffy coat preparation	15	321	21.4	4.1	
No. concentration	2	1210	23.6	3.5	

[a] Total of 28 patients
[b] Mean values
[c] Final incubation concentration

peripheral granulocyte counts to values over 500/mm³. This range does not markedly differ from other groups undergoing BMT without marrow incubation treatment. Two patients did not receive a sufficient engraftment. One of these patients was one-way HLA-D different and died very early on day 21 with septicemia. In the other case, the HLA-D compatibility could not be clearly documented. This patient also showed persisting leukemia at autopsy, factors that may have prevented a sustained engraftment.

In nine out of 28 cases clinically manifest GvH reactions were detected. In five cases GvHD was restricted to skin reactions grade I–II. The skin reactions occurred between day 11 and day 16, persisting for 8–14 days, except two cases with delayed regression. A sixth case showed a grade I liver reaction in addition. In two cases more severe mainly chronic GvHD of skin and liver, reaching grade II–III, was seen. In a third case the course was complicated by manifestation of GvHD on the gut (grade III). Although these patients responded well to a treatment with corticosteroids, chronic GvHD could not be completely suppressed and all three patients died later from infections. Table 4 summarizes the survival an final outcome of the transplanted patients. Two patients died without sustained engraftment on day 20 and day 34 after transplantation of lethal infections. Six patients had a leukemic relapse and died because of this complication between day 106 and 500 posttransplantation. All six patients were cases transplanted after relapse. Three patients died of infections and interstitial pneumonitis (IP). In one case the IP was caused by infection with *Pneumocytis carinii*. None of the patients with IP showed any sign of GvHD. Two patients died of other infections during persisting chronic GvHD. One patient is alive with a testicular relapse. Ten of the 28 patients are alive in complete remission between day 15 and day 26 posttransplantation.

So far our human studies have concerned almost only HLA identical MLC nonreactive leukemic siblings, a situation with a still relatively high probabilitiy for GvHD. In sex-different patients (11 out of the 28 patients reported here) bone marrow has been reported to cause GvHD more frequently [7]. Sex difference appears to influence GvHD also in dogs [8]. The formal proof that ATCG prevents GvHD in MHC-identical patients requires, of course, a greater number of patients than have been listed in this report and should be confirmed in a randomized study. So far our data indicate that ATCG did not interfere with hemopoietic engraftment at dilutions known to be toxic for T cells. The encouraging results in several MHC incompatible animal models should provide impetus for elucidating chances and limitations of such an approach in situations with some degree of MHC incompatibility. Recent mouse experiments have shown in highly H-2 incompatible combinations that an effect of ATCG on GvHD may be further duplicated by addition of complement during in vitro incubation [2, 9].

Table 3. Donor-recipient differences, engraftment, and occurrence of GvHD in 28 patients receiving ATCG-incubated marrow grafts

Donor-recipient differences (No. of patients)			
HLA-D[a] 2/28	Major blood group 5/28	Minor blood group 6/28	Sex 11/28
Engraftment			
BM recovery 26/28	No. take 2/28	Day posttransplant 17±3	Range (days) 12–26
GvHD (No. of patients)			
Clinical grade I–II 6/28[b]	Clinical grade III 3/28	Clinical grade IV 0/28	

[a] Questionable in two other cases
[b] No histological confirmation in one case

Table 4. Fatal complications and survival in 28 patients after transplantation of ATCG-incubated marrow grafts

Patients died Cause of death	No. of patients	Survival time (relapse)[a]
No engraftment	2	20, 34
Leukemic relapse	6	106(83), 126(112), 198(123), 215(181), 233(168), 500(482)
Interstitial pneumonitis and infection	5	33, 38, 39, 64, 71
Acute GvHD	–	–
Infection during chronic GvHD	3	95, 211, 420
Other	1	312

Patients surviving Leukemia type	Status	Survival time[a]	Outcome[b]
ALL	3rd relapse	>1511	CR
AML	2nd remission	> 859	Testicular relapse
AML	1st remission	> 570	CR
AML	2nd relapse	> 532	CR
ALL	4th relapse	> 511	CR
ALL	2nd remission	> 332	CR
ALL	2nd remission	> 208	CR
AML	2nd remission	> 145	CR
AML	1st remission	> 75	CR
CML	Chronic phase	> 54	CR
AML	3rd remission	> 26	CR

[a] Days posttransplantation
[b] CR, Complete remission

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Graft-Versus-Host Disease: Immunobiological Aspects*

P. J. Tutschka

Graft-versus-host disease continues to be a major problem in clinical allogeneic bone marrow transplantation. Despite great efforts to describe the disease clinically, histopathologically and immunologically we are far from understanding the immunobiology of graft-versus-host disease and transplantation tolerance, preventing us from developing rational clinical strategies to overcome this stumbling block.

In allogeneic bone marrow transplantation the lymphohematopoietic system of an adult, fully immunocompetent donor individual is transferred into a recipient that has been rendered immunologically incompetent, and the new graft is expected to survive, to function in an immunologically competent way yet to incorporate the transplantation antigens of the host into the already established repertoire of self, thus achieving a state of specific immunologic tolerance against the host [1–3]. Amazingly enough, such a seemingly impossible task can be achieved, and stable, immunologically competent yet specifically host-alloantigen tolerant chimeric states are reached in clinical and experimental bone marrow transplantation. However, in the majority of cases the complex and poorly understood process of "tolerization" is disturbed and the desired goal not achieved, resulting in the syndromes of acute and chronic graft-versus-host disease. Thus, despite almost 2 decades of clinical bone marrow transplantation, graft-versus-host disease in its acute and chronic form continues to be the major complication in marrow transplantation and a challenge for the clinician and the immunobiologist alike [4–7].

Graft-versus-host disease, to use a very simplistic operational definition, is considered to be the result of immunocompetent T-lymphocytes of donor origin that attack certain target organs of the host [8, 9]. Simonsen [10] and later Billingham [11] were the first to identify the requirements for a graft-versus-host reaction, a view that, at least superficially, is still valid for its practical implications:

1. The graft must contain immunologically competent cells.
2. The host must possess important transplantation isoantigens that are lacking in the graft donor, so that the host appears foreign to it and is therefore capable of stimulating it antigenically.
3. The host itself must be incapable of mounting an effective immunological reaction against the graft, i.e., the graft must have some security of tenure.

Based on that concept intensive research of more than 2 decades has attempted to establish the immunobiology of graft-versus-host reactions (GVHR), graft-versus-host disease (GVHD), and transplantation tolerance. In summary, the following statements were made:

1. The strength and kinetics of GVHR and GVHD are positively correlated with the degree of histoincompatibility.
2. The primary lesions of GVHD are the result of T-cell mediated cytotoxicity to target cells caused by the small, immunocompetent postthymic lymphocytes in the donor marrow inoculum.

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3. GVHR and GVHD are self-terminating immunologic events through deletion of host-responsive clones of donor cells.

Quite in accordance with these postulates were the results of intensive research into the immunobiology of marrow grafting that showed that the magnitude and kinetics of the graft-versus-host reactions (GVHR) were correlated with the degree of histoincompatibility between donor and recipient [1]. Transplantation tolerance, evolving after the resolution of graft-versus-host reactions and disease and characterized by immunologic unreactivity of donor cells toward host, but not third party alloantigens [3], developed rarely, in particular in mismatched donor-recipient combinations, and when it developed it was the result of a deletion or irreversible inactivation of specific clones of donor immunocompetent cells that were reactive against host alloantigens [7].

However, over the years several disconcerting observations [12, 13] were reported that cast some doubt on the validity of a concept that seemed so well founded by a plethora of experimental and clinical data. Recipients of marrow grafts that were mismatched not in major but minor histocompatibility antigens displayed graft-versus-host reactions and disease that were virtually indistinguishable in magnitude from those seen in major mismatches, a finding observed in humans, dogs, and rodents [14–17]. Even recipients of syngeneic grafts were described that showed graft-versus-host disease [18–21]. Furthermore, animals that were inbred and presumably of identical histocompatibility makeup showed marked differences in the incidence and severity of GVHD when grafted with marrow from the same histoincompatible inbred donor strain, some animals displayed no signs of GVHD clinically and histologically while others showed fatal GVHD [22]. Moreover, germ-free murine recipients of histoincompatible marrow showed no GVHD but developed the disease after intentional contamination with certain microorganisms [23, 24], suggesting that GVHD was not solely dependent on the degree of histoincompatibility between donor and host but rather required a trigger, most probably a mircoorganism. Viruses, in particular herpes type virus, a common complicating factor in clinical GVHD [19–29], have been incriminated in this trigger process, a postulate supported by the circumstantial evidence that GVHD could developed after a long lag period (up to 70 days in humans) and long after a seemingly complete and functioning lymphohemapoietic graft had been established [4, 8, 12]. These findings, seemingly inconsistent with the classical concept, led us to hypothesize that the complex immunological situation post-marrow grafting was not only dependent on the immunogenetically determined killer cells but on a counterforce of suppressor cells [3, 30, 31], that the magnitude and kinetics of the cytotoxic response were not determined by the antigeneic difference but by the magnitude of a suppressor cell response developing together with the cytotoxic cells. Transplantation tolerance, then, would not be seen as the result of a clonal deletion of alloantigen responsive cells but rather as a delicate balance between cytotoxic and suppressor cells whereby ultimately the suppressor cell arm gains preponderance, resulting in the stable, tolerant chimera [30–32].

Over the past several years we were indeed able to show that the specific immunologic tolerance seen in stable chimeras was maintained by alloantigen-specific suppressor cells [33]. This suppressor cell system has been rather extensively characterized in our rat bone marrow transplant model. Histoincompatible (mismatched at the Rt 1 locus) bone marrow allografts were established in lethally irradiated rats. At various times after transplantation lymphoid cells were harvested, subjected to mixed lymphocyte cultures, and assayed for immunological tolerance and for suppressor cells in vitro and in vivo. Alloantigen nonspecific suppressor cells appeared in the chimera 40 days after grafting, coinciding with the resolution of graft-versus-host disease. When specific tolerance was finally achieved in vivo and in vitro, a process that required between 100 and 250 days, the nonspecific suppressor cells were replaced by nylon wool adherent T-lymphocytes that specifically suppressed host alloantigen responses and could adoptively transfer the suppression of GVHD, suggesting that indeed the balance called operational tolerance

was actively maintained by specific suppressor cells. Specific tolerance in the chimeras was maintained during the 2 years of follow-up; however, the numbers of suppressor cells declined until they could no longer be demonstrated in vitro. A complete clonal deletion of host-reactive cells, though, of either alloresponsive clones or suppressive clones, did not occur. Restimulation of suppressor cells was possible with host antigen either by adoptive transfer or by inoculation of chimeric animals, suggesting that a clonal reduction had taken place in the long-term chimera which was followed by an induced expansion of suppressor cells clones [34].

The above described experiments suggested that alloreactive clones of cytotoxic cells were present in chimeras, but operationally not expressed, a postulate supported by the results of fractionation studies. Spleen cells from long-term complete and stable chimeras that were specifically tolerant to host alloantigens in vivo and in vitro were passed through nylon wool columns. The nylon wool nonadherent cells were then stimulated with host type stimulator lymphocytes and regained their ability to proliferate and develop specific cytotoxic effector cells [34].

In summary, these data seemed to indicate that induction of transplantation tolerance required a complex process of killer and suppressor cell interactions ultimately resulting in the incorporation of previously "foreign" antigens into the repertoire of "self".

It was conceivable to assume that not only transplantation tolerance but also tolerance against self was the result of a similar mechanism. This meant that in an adult organism autoreactive potential killer cells were present which were prevented from proliferating by the action of autoreactive thymus-dependent suppressor cells, which like in the long-term allogeneic chimera were present in a clonally reduced state.

If that were the case there should be circumstances under which a true autoaggression against transplantation antigens would occur, where a syngeneic or autologous reaction could be demonstrated that was neither clinically nor histologically distinguishable from a graft-versus-host reaction.

Such heretic thoughts have been expressed before. Cohen et al. reported about autosensitization in vitro [35], Parkman et al. [36] identified a subpopulation of lymphocytes in human peripheral blood cytotoxic to autologous fibroblasts and later explained the lack of autoreactivity in murine spleen cells by the concomitant presence of suppressor and cytotoxic lymphocytes, a view supported by the studies of L'age- Stehr and Diamantstein [37]. Gozes et al. finally induced a "syngeneic GVHR" in popliteal lymph nodes by spleen cells of old C57 B1/6 mice [38].

Clinically severe graft-versus-host disease has been described repeatedly in recipients of syngeneic bone marrow grafts [18–21]. Although apparently a rare occurrence in clinical transplantation, the reports suggested that "syngeneic GVHD" was neither an oddity nor the result of a transfusion accident (e.g., the infusion of an unirradiated blood product). Moreover, the factual presence of a "syngeneic GVHD" suggested that such a situation could be explored to understand better the nature of self-tolerance and disease states where self-tolerance was disturbed.

The bone marrow inoculum to be transplanted into a syngeneic, lethally irradiated recipient should contain autoreactive cytotoxic as well as autoreactive suppressor cells. Removal of suppressor cells by appropriate separation techniques prior to marrow infusion should result in syngeneic GVHD. Indeed, when suppressor cells were removed by either nylon wool fractionation or chemoseparation with 4-hydroxyperoxyclyclophosphamide we were able to create even syngeneic GVHD in lethally irradiated rat recipients [39, 40].

Acute graft-versus-host disease, then, could be seen as a disturbance of the tolerization process where either the cytotoxic effector arm is enhanced or the suppressor arm is either diminished or absent, leading to the observed injuries.

Chronic GVHD, in contrast, appears to present as an even more complex immunobiological situation. Rats with chronic GVHD, when evaluated immunologically, show an immunodeficiency, primarily of the T-cell arm, resulting in prolonged survival of third party skin grafts, depression of the antibody response to sheep red

blood cells, and impairment of the proliferative response of lymphocytes to alloantigens of host and third party strains. When assaying for suppressor cells we found that spleen and peripheral blood contained abundant numbers of alloantigen nonspecific suppressor T-lymphocytes that suppressed proliferative responses of original donor type to original host and third party alloantigens [41–45]. Adding such spleen cells to normal donor marrow inocula prior to transfer into secondary hosts not only prevented the development of acute GVHD but led to the rapid establishment of chronic GVHD clinically and histologically within 4 weeks after cell transfer. Speculating that nonspecific suppressor cells were causally involved in the pathogenesis of chronic GVHD, we harvested nonspecific suppressor T-cells from the spleens of healthy bone marrow chimeras early (48 days) after transplant and added them to normal donor marrow inocula prior to transfer into secondary hosts. Again, acute GVHD was prevented, but chronic GVHD developed within 4 weeks. Finally, we implanted thymuses from rats with chronic GVHD into normal rats that were lethally irradiated and reconstituted with donor type marrow immediately before thymus implantation. Those animals not only developed acute GVHD but also chronic GVHD within the first 4 weeks of marrow grafting [22, 34, 44, 45].

Thus chronic GVHD, at least in the rat model, again seemed to represent an imbalance of immunologically active cells, but unlike acute GVHD, not a relative or absolute decrease in the number of alloantigen-specific suppressor cells, but rather an increase in the number of alloantigen nonspecific suppressor cells under the influence of a malfunctioning thymus.

These concepts developed in animal models have been examined in the clinical bone marrow transplant situation, and some new strategies for the prevention and treatment of GVHD have been suggested. Patients who received an allogeneic bone marrow transplant, engrafted successfully, and have no evidence of GVHD, do indeed show suppressor T cells specific for host alloantigens [46]. Patients with acute GVHD lack those suppressor cells, whereas patients with chronic GVHD show large numbers of alloantigen nonspecific suppressor cells [46, 47].

Attempts to engineer the immunobiological situation after marrow transplantation and to facilitate the development of specific suppressor cells are encouraging. An agent of particular interest for this task is cyclosporin A, a fungal polypeptide that in vitro prevents the maturation of cytotoxic effector cells yet permits the development of suppressor effector cells [48, 49]. After very successful animal studies [49, 50], this agent is now used clinically to prevent acute GVHD. A pilot study performed in our institution has shown that this promising agent indeed prevented clinically severe GVHD in a majority of patients [51]. However, the study has also identified side effects of the new agent leading to clinically relevant complications, in particular, renal failure.

It is hoped that a better understanding of the pharmacology of this agent will lead to an improved utilization of the agent clinically, as well as further our understanding of transplantation tolerance in the recipient, thus widening the clinical applicability of bone marrow transplantation.

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T-Cell Phenotypes in Mixed Leukocyte Reactions and After Bone Marrow Transplantation: Are Ia-Antigen on T-Cells a Marker for GvH Reactions?

K. Stünkel, C. Bender-Götze, B. Netzel, and E. Thiel

A. Introduction

Bone marrow transplantation (BMT) offers an alternative in the treatment of lethal blood disorders. Cells of the restored lymphoid system are then of donor origin. There is a high incidence of lethal infections (approx. 25%) during the first 4 months following BMT, presumably due to a pronounced cellular and humoral immunological deficiency [6, 16]. Graft-versus-host disease (GvHD) is also a major problem associated with BMT. Both acute and chronic forms of GvHD are characterized by severe combined immunoincompetence. Although the pathophysiological mechanism at the cellular level remains largely unresolved, it is widely accepted that alloreactive T cells are involved in the appearance of GvHD [7, 13, 14]. There is also evidence that disturbances in the regulatory pathways between T-cell subclasses may result, at least partially, in immunodeficiencies and GvHD [11, 12]. In addition, it has been described that Ia antigens on T-cell blasts occur after allogeneic and mitogenic activation [8]. Therefore, it is not surprising that in disorders related to T-cell subsets the appearance of Ia-antigens was observed [11].

Thus, the appearance of Ia-like determinants on activated human T cells may provide a marker for monitoring T-lymphocyte reactions in vitro and in vivo. Therefore, the Ia antigen may be diagnostically relevant for the recognition and monitoring of GvH following BM grafting. To study the relevance of the Ia marker on T cells, we investigated peripheral blood of nine recipients of marrow allografts and the progress of T-cell reconstitution, as well as the distribution patterns of the T-helper and the T-suppressor cells.

B. Methods

I. Patients

The study was performed on nine patients following bone marrow transplantation for acute leukemia (four patients) and aplastic anemia (five patients). Of the nine patients one had chronic GvHD and one had acute GvHD, which contributed to mortality. No evidence of acute or chronic GvHD was documented in sex patients. Two other patients died of non-GvHD problems. Of the four patients with acute leukemia two (S.M. and K.M.) received an engraftment of bone marrow of HLA-MLC matched siblings preincubated with anti-T-cell globulin (ATCG) (for details see Rodt et al. [13]) and one (F.M.) with anti-cALL globulin (AcALLG) treated autologues bone marrow (for details see Netzel et al. [9]). In addition, a 4-month-old baby (K.A.M.) was studied, who developed an acute GvH reaction following blood exchanges from its mother. Two patients with acute lymphatic leukemia who were in an early phase of remission were included to examine Ia expression on T cells.

II. Isolation of Lymphocytes and Stimulation Assays

Membrane marker analysis and cultures for mixed leukocyte reaction (MLC) were performed with the total mononuclear frac-

tion of the Ficoll-Hypaque gradient isolated peripheral blood cells; 0.5×10^6/ml in a 1:1 ratio of responder : irradiated (3000 rad) stimulator cells were cultured in RPMI 1640 with 10% human AB serum.

III. Polyclonal and Monoclonal Antibodies Used for Surface Marker Analysis

Polyclonal antisera directed against human Ia-like antigens were purchased from Alpha Gamma Labs (Sierra Madre, United States). The reagents OKT3, OKT4, OKT5, OKT6, OKT8, OKT9, OKT10, and OKM1 (generous gifts of Dres. G. Goldstein and P. C. Kung, Raritan, United States) have been previously characterized in detail [10]. The second antibody for indirect immunofluoresence was a goat anti-mouse IgG-FITC and a goat anti-rabbit IgG-TRITC (Tago Inc., Burlingame, United States).

C. Results

The mixed leukocyte culture (MLC) was used as a test in vitro system for the in vivo GvH reaction which sometimes occurs as a consequence of BMT. The comparison of ^{3}H-thymidine uptake and Ia-expression in a kinetic study correlates in that only in the HLA-MLC different situation does a high proliferation (cpm) and a high percentage of Ia-positive cells occur (as much as 60%). In addition, ^{3}H-thymidine labeling indices and Ia-antigen expression in MLC were compared in a family whose members varied in LD determinants to various degrees. Depending on the extent of the LD difference, distinct stimulation indices (STI) and blast cell counts were obtained. Simultaneous determinations of Ia-positive cells by immunofluorescence revealed a good correlation of the number of positive cells and the STI (data not shown).

Additional phenotypical characterization by monoclonal antibodies of the OKT series in MLC using OKT10 and OKT9 (both thymic associated, but not T-lineage specific) and OKT6 (intrathymic) showed a great increase ($\leqq$90%) of T10$^+$ cells on days 6–7 including all blasts and even small cells. In contrast, OKT9 reactive cells are maximally expressed at day 5 (up to 45% of the blast cells). No blast or small cell was found to be positive with OKT6. Using the functional defined antibodies OKT4, OKT5, and OKT8, only the proportion of OKT4 and OKT8 positive cells was slightly augmented. Generally, the ratio of OKT5 : OKT4 and OKT8 : OKT4 was stable (data not shown).

Table 1 presents the data of a continuous study (patient S. M.) up to 434 days following BMT. A remarkable observation was that this patient, who was *without* any GvHD, displayed a high number of T cells expressing Ia antigens. Even after 434 days 9% of Ia-presenting T cells were found. The same observation was made in three other patients free of GvHD (Fig. 1) during a period of 105 days following BMT. A normal situation (except in patient K. C., perhaps due to a virus infection) was found in the blood of three patients 2½ years or more after BMT compared with healthy controls (Table 2, Fig. 1).

In patients *with* GvH reactions no contrary results were obtained concerning the percentage of Ia expression (Fig. 1), even in the cases with an acute reaction. On the basis of the findings in MLC, one would expect this high percentage of Ia-positive cells in this group of patients.

Within 4 months of transplantation the percentage of T cells (OKT3$^+$) in patients S. M. and J. C. had already reached normal values. The T-cell reconstitution in patient K. M. (data not shown) and F. M. (Table 2) remained retarded when compared with S. M. It is very likely that within the next 1 or 2 months the relative T-cell count would be normalized.

Regarding the T-cell subpopulations, time-dependent changes in the percentage of OKT4$^+$ and OKT8$^+$ lymphocytes were observed post-BMT (Table 2). Shortly after marrow engrafting (less than 2 months) the OKT5 and OKT8 subset was two times greater than normal (both are markers for suppressor/cytotoxic T cells, although OKT8 binds to about 5% more lymphocytes than OKT5), whereas the OKT4-helper/inducer subset was only 50% of that found in normal blood (patient S. M.). As soon as 2 months following BMT the T4 : T5 ratio was approximately 1 : 1 (patients S. M. and F. M.). This ratio subsequently returned to its normal state of

Days after BMT	OKT3	T4	T5	T8	T10	Ia
	% positive cells					
8	44					39
14	65				57	34
20	54				58	21
28	51	20	37	45	68	29
42	52	30	38	50	58	26
70	61				56	21[a]
82	56	31	34		59	33[a]
335	52	30	22	26	46	11[a]
434	49	32	17	24		9[a]
NP[b] ($n=6$)	65±10	40±8	18±7	23±8	14±5	13±4

Table 1. Antigenic pattern shown by the mononuclear cells of an 8-year-old child (S.M.) at various times after bone marrow transplantation; clinically no evidence of GvH activity

[a] T3 + Ia = double membrane marker analysis
[b] Controls

2 : 1 (S.M.). It has also observed that the percentage of OKT8 was increased and often much higher than that obtained with OKT5. This was most apparent when the T4 : T5 ratio was 1 : 1.

In patients with GvHD the ratio of OKT4 : OKT5 : OKT8-positive cells was not the same as in non-GvHD patients. Even after 15 months B.G., who had a mild form of a chronic GvHD, still showed an equal percentage of T4 : T5 carrying cells, while $OKT4^+$ cells were within the normal range. After 2 months of receiving chemotherapy the T-cell count ($OKT3^+$) was decreased, as were all subsets. Interestingly, this was similar to a post-BMT situation

Patient		OKT3	T4	T5	T8
		% positive cells			
< 2 years after BMT					
S.M.	28 days after BMT	51	20	37	45
	42 days after BMT	52	30	38	50
	82 days after BMT	56	31	34	
	335 days after BMT	52	30	22	26
	434 days after BMT	49	32	17	24
F.M.	58 days after BMT	21	12	14	22
J.C.	(acute GvH)				
	122 days after BMT	61	12	52	60
B.G.	(chronic GvH)				
	15 months after BMT	66	43	45	64
	17 months after BMT	49	18	31	48
> 2 years after BMT					
H.R.	2½ years after BMT	76	56	26	59
K.C.	4 years after BMT	54	30	18	26
M.C.	6½ years after BMT	57	36	26	28
NP[a] ($n=6$)		65±10	40±8	18±7	23±8

Table 2. T cells and T-cell subpopulations present in bone marrow transplant patients at various times after transplantation

[a] Controls

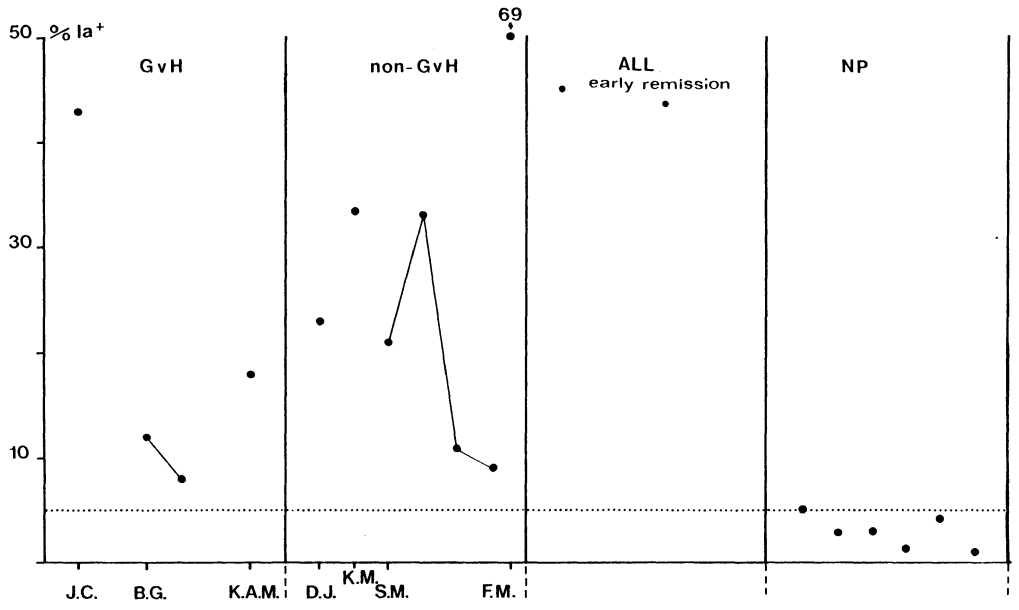

Fig. 1. Ia-like antigen expression on T cells of patients with and without GvH reaction (up to 2 years after BMT) compared with normal

(S.M., day 28). Patient J.C., who had an acute GvH reaction, demonstrated a similar situation 122 days after BMT, but with a very low $T4^+$ cell count (12%) and a very high $T5^+$/ $T8^+$-cell count (52%/60%).

Very low or negative results were obtained using OKT6 and OKT9. OKT10 reacted in all cases studied with a remarkable increased percentage, both with or without GvHD.

D. Discussion

Mixed leukocyte cultures are a possible in vitro test system which, within certain limits, may be comparable to BMT-induced GvHD. We were able to demonstrate newly appearing antigenic determinants of T-cell blasts after allogeneic HLA-different activation by means of a panel of monoclonal antibodies and Ia-like specific antisera. These new determinants are recognized by the OKT10, the OKT9, or the Ia antibodies. In HLA-identical or autologous cultures no blasts nor any additional Ia and OKT10/T9-positive cells were found.

In contrast to the in vitro data as well as data recently published by Reinherz et al. [12] and De Bruin et al. [5], we found remarkably high percentages of Ia antigen carrying T-lymphocytes independent of the occurrence of GvH reactions. Therefore, no difference was found between patients with or without GvHD, which is in agreement with Atkinson et al. [1]. However, a time-dependent reduction in the expression of Ia antigens on the chimeric T cells toward normalization was observed in non-GvHD patients. We also found that approximately 50% of T cells from two ALL, early postchemotherapeutic remissive patients expressed Ia determinants and about 70% of T cells of an autologous graft.

We conclude that the demonstration of Ia-like antigens in a given period of time following BMT is due to an activation caused by the reconstitution of bone marrow and other lymphoid tissues. An additional possibility for the explanation of our results could be the fact that Ia-antigens appear on T-cells not only after allogeneic stimulation but also after stimulation with other antigens (viral) and mitogens, observed in vitro and in vivo. Thus, the expression of Ia does not seem to be a suitable marker for the detection of GvH reactions that arise during the early stages following BMT.

The detection of a substantial portion of OKT10 binding cells is probably not related to immature T-lymphocytes as presumed by De Bruin et al. [5]. The results in MLC and in combined staining with OKT10+OKT3 or OKT10+OKM1 (OKM1 reacts with monocytes [4] and most NK subsets [15]) in samples of some patients indicate that the presence of this antigen could be caused by activation (data not shown). With this combined staining we found that most of the $OKM1^+$ cells also reacted with OKT10. This seems to be reasonable with regard to our results on activated monocytes with OKT10 [3]. The remaining percentage of $T10^+$ cells was comparable to the T-cell fraction, which was stained by anti-Ia antisera.

The imbalance in T-lymphocyte subsets in the early stages after BMT or in patients with GvHD was also confirmed by other investigators [1, 2, 5]. But in contrast to Reinherz et al. [12], we cannot support the view that acute GvHD is predominated by cells of helper/inducer phenotype (TH_2^-), defined by heteroantisera.

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Monoclonal Antibody Therapy: "Model" Experiments with Toxin-Conjugated Antibodies in Mice and Rats

P. E. Thorpe, S. I. Detre, D. W. Mason, A. J. Cumber, and W. C. Ross

A. Introduction

The advent of cell fusion techniques for producing monoclonal antibodies has stimulated world-wide effort in the search for antibodies with specificity for neoplastic cells. In parallel, a number of laboratories have attempted to devise ways of attaching cytotoxic agents to antibodies with the aim of generating potent anticancer agents from monoclonal antibodies that exhibit sufficient specificity for cancer cells.

The most successful ploy for arming antibody molecules has been to couple them to highly poisonous toxins such as abrin, from the jequirity bean, and ricin, from the castor bean. Abrin and ricin are glycoproteins comprising two polypeptide subunits, A and B, joined by a disulphide bond. The B-chain binds to galactose-containing molecules which are to be found on most cell surfaces and the A-chain is believed to penetrate the plasma membrane or the membrane of an endocytic vesicle and kill the cell by damaging ribosomes (reviewed in [1]).

B. Anti-tumour Effects of Antibody-Abrin and Antibody-Gelonin Conjugates

In the first series of experiments, $Thy_{1.1}$-expressing lymphoma cells growing in mice or in tissue culture were attacked with the $F(ab')_2$ fragment of monoclonal anti-$Thy_{1.1}$ antibody (IgG_{2a}) coupled to abrin. The antibody-toxin conjugate and a control conjugate made with the $F(ab')_2$ fragment of normal murine IgG_{2a} were prepared using a mixed anhydride derivative of chlorambucil as the coupling agent as described previously [2]. The simplest conjugates, consisting of one molecule of $F(ab')_2$ and one of abrin, were purified. No loss in the binding capacity of the anti-$Thy_{1.1}$-abrin to antigens upon AKR-A and BW5147 lymphoma cells was apparent from indirect immunofluorescence analysis in 100 mM lactose [3].

In vitro, the anti-$Thy_{1.1}$-abrin conjugate was a very effective and moderately specific cytotoxic agent for AKR-A and BW5147 cells (Fig. 1). Treatment of the cells with the conjugate at 2.5×10^{-11} M sufficed to reduce their capacity to incorporate ^{3}H-leucine into protein by 50%. The cytotoxic action of the conjugate upon BW5147 cells was similar in potency to that of unconjugated abrin, whereas with AKR-A cells the native toxin was about tenfold more effective. The cytotoxic effect of the conjugate was specific, as shown by the comparative ineffectiveness of the control conjugate which reduced the leucine incorporation of the BW5147 and AKR-A cells by 50% at 2.5×10^{-9} M and 5×10^{-10} M respectively.

The therapeutic value of anti-$Thy_{1.1}$-abrin was assessed against AKR-A and BW5147 cells growing in T-cell-deprived CBA mice. The $Thy_{1.1}$ antigen expressed by the lymphoma cells is not found in CBA mice and so it constituted a tumour-specific antigen in this model system. When 10^5 AKR-A cells were injected intraperitoneally, they grew progressively, initially as an ascitic tumour, and killed the mice between 18 and 21 days later. Treatment of the mice with 1.5 pmol anti-$Thy_{1.1}$-

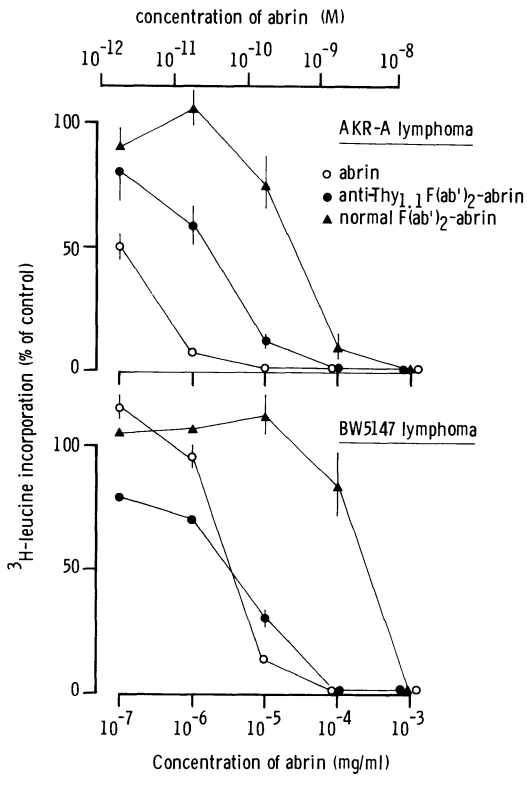

Fig. 1. The cytotoxic effects of abrin alone (○) and of conjugates with the *F(ab')*$_2$ fragments of monoclonal anti-Thy$_{1.1}$ antibody (●) or of normal mouse IgG$_{2a}$ (▲) upon AKR-A and BW5147 cells in tissue culture. The cells were treated for 1 h at 37 °C with abrin or the conjugates and then were washed and 23 h later 1 μCi ^{3}H-leucine was added to the cultures. The ^{3}H-leucine incorporated during a 24 h period is expressed as a percentage of that in untreated cultures. Vertical lines represent one standard deviation on the geometric mean of triplicate determinations. Treatment of the cells with unconjugated anti-Thy$_{1.1}$ *F(ab')*$_2$ at concentrations as high as 10^{-7} *M* did not alter their rate of leucine incorporation

abrin administered intraperitoneally 1 day after the lymphoma cells extended the median survival time of the animals by 5.5 days (Fig. 2). Neither 1.5 pmol anti-Thy$_{1.1}$ *F(ab')*$_2$ alone, nor abrin at a dose corresponding to half the LD$_{50}$, prolonged the survival time of the animals. Experiments in which graded numbers of untreated AKR-A cells were injected intraperitoneally into T-cell-deprived mice established that an extension in median survival time of 5.5 days was approximately that which resulted from a 100-fold reduction in the number of tumour cells injected, suggesting that the anti-Thy$_{1.1}$-abrin conjugate had eradicated 99% of the lymphoma cells. This deduction was supported by the finding in a further experiment that 40% of animals which received 10^3 tumour cells (equivalent to 100 lethal doses, since about ten cells are needed to kill an animal) were cured by their treatment with the conjugate. However, when the conjugate was administered intravenously rather than intraperitoneally, no anti-tumour action was observed, as was also the case when established subcutaneous tumours were attacked. Thus the therapeutic activity of the conjugate was disappointing and was only apparent when small numbers of tumour cells were attacked with conjugate delivered directly to the site of tumour growth.

The problem with conjugates containing intact toxins is that they are highly poisonous to animals, probably because they bind non-specifically to cells through the galactose-binding site on the B-chain of the toxin moiety. This has the important corollary that conjugates injected intravenously might be expected to form semi-stable complexes with glycoproteins free in the plasma or upon the surface of erythrocytes. This could delay the diffusion of the conjugate out of the bloodstream, a notion consonant with the observation above that intravenously administered conjugate was ineffective. One way around these problems is to link antibodies directly to the isolated toxin A-chain. Alternatively, use could be made of one of the virtually non-poisonous inhibitors distributed widely in the plant kingdom whose action upon eukaryotic ribosomes is apparently identical to that of ricin A-chain (reviewed in [4]). One such inhibitor is gelonin, from the seeds of *Gelonium multifluorum* [5].

Accordingly, monoclonal anti-Thy$_{1.1}$ antibody was coupled to gelonin, using the SPDP reagent which introduces a disulphide linkage between the two protein molecules [6]. The conjugate was, however, only weakly cytotoxic to AKR-A or BW5147 cells in tissue culture, a concentration of 10^{-7} *M* being needed to reduce the leucine incorporation of the cells by half. This result contrasts with the extremely potent and

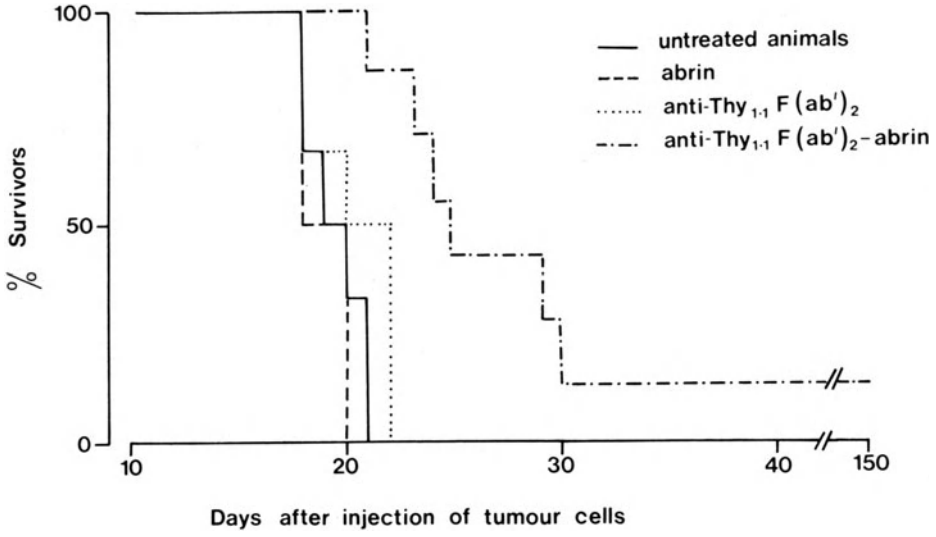

Fig. 2. Prolongation of survival of immunologically deficient CBA mice bearing a $Thy_{1.1}$-expressing lymphoma following the administration of anti-$Thy_{1.1}$ *F(ab')2*-abrin. The mice were injected intraperitoneally with 10^5 AKR-A lymphoma cells and 1 day later received an intraperitoneal injection of 1.5 pmol anti-$Thy_{1.1}$ *F(ab')2*-abrin (–·–·–), 1.5 pmol of unconjugated anti-$Thy_{1.1}$ *F(ab')2* (·····) or 0.15 pmol of unconjugated abrin (– – –). The mice were rendered T-cell deficient by a procedure of thymectomy, whole body irradiation and reconstitution with normal CBA bone marrow

specific inhibitory action of the same conjugate upon resting AKR T-lymphocytes in tissue culture [6] and typifies the variable effectiveness of conjugates containing toxin A-chain that has been observed in many laboratories (reviewed in [7, 8]). Nevertheless, when injected intraperitoneally at dose levels of 700 pmol (corresponding to less than 1/50 of the minimal lethal dose for free gelonin) the conjugate prolonged by 1 week the median survival time of CBA mice bearing intraperitoneal AKR-A lymphoma cells.

Several factors could operate in animals to prevent antibody-toxin conjugates from exerting anti-tumour activity of a potency and selectivity predicted by in vitro experiments. Firstly, intact abrin and ricin, ricin A-chain and gelonin are glycoproteins containing mannose and *N*-acetyl glucosamine residues and, by analogy with similar molecules, are expected to be withdrawn from the blood circulation by the reticuloendothelial system which is equipped with receptors for these sugars [9, 10]. Secondly, it could be that the chemical linkage used to form the conjugate breaks down in animals; there is evidence that disulphide linkages are prone to cleavage by reduction or disulphide exchange with thiol-containing molecules [11]. Lastly, it is not known how easily conjugates diffuse out of the bloodstream to their intended site of action. Until the importance of these factors is assessed and countermeasures devised to those which prove problematical, the potential of antibody-toxin conjugates as anticancer agents will remain undetermined.

C. Selective Killing of Malignant Cells in Leukaemic Bone Marrow in Vitro

The treatment of leukaemia patients with high-dose chemotherapy, total body irradiation and allogeneic bone marrow transplantation runs the risk of provoking life-threatening graft-versus-host reactions and rejection of the marrow graft. These problems would not arise if the patient's own bone marrow, extracted before radiochemotherapy, could be treated with an antibody-toxin conjugate to destroy the malignant cells which had infiltrated it and then injected back into the patient on completion of the treatment.

To explore this possibility, we adopted a model system in which a mixture of 10^3 or 10^4 rat T-cell leukaemia cells and 10^7 bone marrow cells was incubated with a conjugate of ricin and the monoclonal antibody, W3/25, washed and injected into 650-rad-irradiated PVG rats [12, 13]. The W3/25 antigen is expressed by the leukaemic cells and by rat T-helper cells, macrophages and thymocytes but is absent from bone marrow stem cells. Toxicity to haematopoietic stem cells was blocked by including lactose in the incubation mixture to antagonise the non-specific binding of the conjugate via its ricin moiety to galactose residues upon their cell surface. The lactose also reduced the quantity of conjugate that bound to erythrocytes and other cells in the inoculum to the level where no signs of toxin-poisoning were seen in the recipient animals.

The bone marrow was acquired from the PVG 1-a strain of rat which is congenic with PVG except that the immunoglobulin light chain genes are derived from the DA strain which expresses the 1-a allotype rather than the 1-b allotype of PVG rats. The survival of haematopoietic stem cells in the leukaemic marrow inoculum treated with the conjugate was measured from their ability to compete with the residual stem cells in the irradiated rat and thus produce B-lymphocytes that expressed the donor (1-a) allotype. As shown in Table 1, there was good, although not complete, preservation of haematopoietic stem cell activity in leukaemic marrow treated with W3/25-ricin at 2.1 μg ricin/ml for 1 h at 37 °C in 100 m*M* lactose. The 1-a chimaerism was between 30% and 46% as compared with 61%–68% in the recipients of untreated marrow cells.

None of the animals that received inocula of 10^3 leukaemic cells incubated with the conjugate developed leukaemia and, of three recipients of 10^4 leukaemic cells, only one did so. Since about ten cells are needed to induce leukamia, it can be calculated that the conjugate had destroyed 99.9% of the malignant cells in the marrow inoculum. This conclusion was supported by other experiments in which animals injected with 10^6 conjugate-treated leukaemic cells were found to develop disease 8–10 days later than recipients of 10^6 untreated cells and at the same time as recipients of 10^3 untreated cells. Neither treatment of leukaemic cells in 100 m*M* lactose with W3/25 antibody alone at 20 μg/ml nor with a control conjugate made with an irrelevant monoclonal antibody, MRC OX8, delayed the appearance of leukaemia in the recipients.

It is concluded that antibodies linked to intact toxins or, as used by Krolick and his colleagues [14], to ricin A-chain, potentially could be used to destroy malignant cells in autologous bone marrow grafts in man.

Table 1. In mixtures of leukaemic cells and bone marrow cells the malignant cells are specifically killed by the antibody-ricin conjugate

Cells injected	Incubation conditions	No. of recipients	Day of appearance of leukaemia	% B-Cell chimaerism
10^3 Leuk. + 10^7 BM	Med.	3	16, 17, 18	–
	Med. + conj.	3	>67, >67, >67	34.0, 37.5, 38.7
10^4 Leuk. + 10^7 BM	Med.	3	16, 16, 16,	–
	Med. + conj.	3	36, >67, >67	–30.6, 46.5
10^7 BM only	Med.	3	–	61.4, 66.2, 67.8

Med., Dulbecco's phosphate buffered saline containing $CaCl_2$, $MgCl_2$ and supplemented with 100 m*M* lactose; conj., W3/25-ricin (M_r 350,000) at a concentration of 2.1 μg ricin/ml and 10 μg IgG/ml. Day of appearance of leukaemia: number of days which elapse after injecting the leukaemic marrow cells before the animal's WBC reached 2×10^4 mm^{-1}. Other details are given by Thorpe [12] and Mason [13].

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In Vitro Cytodestruction of Leukemic Cells in Human Bone Marrow Using a Cocktail of Monoclonal Antibodies*

T. W. LeBien, R. C. Ash, E. D. Zanjani, and J. H. Kersey

Therapy for human leukemia is currently limited by toxicity of chemoradiotherapy to normal bone marrow stem cells. This limitation can be circumvented by the prior removal and subsequent reinfusion of autologous bone marrow [1, 2]. In order for successful engraftment to occur, reinfused bone marrow must be pruged of leukemic cells ex vivo while the viability of stem cells remains intact. Monoclonal antibodies have the potential of providing this selective destructive effect.

During the past several years our laboratory has produced and characterized a panel of monoclonal antibodies recognizing cell surface molecules *primarily* expressed on leukemic cells and B cells [3–7]. We now present preliminary experiments designed to test the feasibility of using monoclonal antibodies BA-1, BA-2, and BA-3 (anti-CALLA) for the ex vivo elimination of leukemic cells in autologous bone marrow transplantation.

A. Materials and Methods

1. Antibodies and Complement

The production, utilization, and characterization of monoclonal antibodies BA-1, BA-2, and BA-3 have been previously described in detail [3, 4, 7]. Baby rabbit complement was obtained from Pel-Freez Biologicals (Rogers, AR).

2. Source of Cells

The established leukemic cell lines NALM–6 (pre-B ALL), REH (non-T, non-B ALL), and KOPN-1 (pre-B ALL) were provided by Dr. Jun Minowada, Buffalo, NY, Fresh leukemic cells were obtained from patients seen on the Pediatric Oncology service at the University of Minnesota. Leukemic cells obtained from bone marrow were separated on Ficoll-Hypaque density gradients and cryopreserved in liquid nitrogen as previously described [8]. Normal bone marrow obtained from adult volunteers was separated on Ficoll-Hypaque density gradients.

3. Stem Cell Assays

Bone marrow stem cell assays to detect CFU-GEMM, CFU-GM, CFU-E, and BFU-E were conducted as previously described [9].

4. Cytotoxicity Assays

Cytotoxicity assays utilizing complement-dependent cytolysis were conducted by trypan blue exclusion [10], or chromium (^{51}Cr) release. Target cells were labeled with sodium chromate for 1 h at 37 °C, washed 3 times, and admixed with normal bone marrow to yield a final leukemic cell/bone marrow cell ratio of 1 : 10, 1 : 100, or 1 : 1000. All cell mixtures were adjusted to 5×10^7 cells (leukemic and normal) per milliliter in RPMI 5% human serum albu-

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min. The various mixtures were then incubated with monoclonal antibodies BA-1, BA-2, and BA-3, all at a final ascitic fluid dilution of 1 : 100, and corresponding to 1–10 μg antibody protein per milliliter of cells. Baby rabbit complement was added to a final dilution of 1 : 6. A negative control consisted of substituting control ascitic fluid for the monoclonal antibodies. All cell populations were incubated in triplicate in 12×75 Falcon polystyrene tubes for 1 h at 37 °C on a rocker platform. The cells were then centrifuged out and a volume of supernatant was removed from all tubes for quantitation of ^{51}Cr-release. Maximum release was accomplished by lysing the ^{51}Cr-labeled leukemic cell/bone marrow cell mixtures with 0.5% NP-40. Percent specific ^{51}Cr-release was then determined using the following equation:
(Exper. – Cont./Max. – Cont.)×100.

B. Results and Discussion

Table 1 outlines the general characteristics of monoclonal antibodies BA-1, BA-2, and BA-3. The three antibodies recognize distinct cell surface molecules; BA-1 recognizing a 30K dalton protein (LeBien, et al., unpublished), BA-2 recognizing a 24K dalton protein [4], and BA-3 recognizing the 100K dalton glycoprotein common acute lymphoblastic leukemia antigen (CALLA) [7]. All three antibodies fix rabbit complement and effectively lyse target cells expressing the individual antigens in vitro. A prominent characteristic of BA-1 and BA-2 is their inability, in the presence of rabbit complement, to inhibited the growth of bone marrow stem cells (CFU-GEMM, CFU-GM, BFU-E, and CFU-E) in vitro [11, 12]. Using rabbit heteroantisera and monoclonal antibody J-5, CALLA has been shown to be absent from bone marrow stem cells [13–15]. Similar results have been obtained with the anti-CALLA monoclonal antibody BA-3 (Ash et al., unpublished). The three antibodies also bind to leukemic cells from the majority of patients with non-T ALL [16]. Thus, BA-1, BA-2, and BA-3 are excellent candidates for elimination of leukemic cells in autologous bone marrow transplantation.

Table 1. General characteristics of BA-1, BA-2, and BA-3

Antibody	Ig class	Antigen detected	Complement fixation	Binding to stem cells	Binding to non-T ALL[a]
BA-1	M	p30 (?)	+	–	80%
BA-2	G3	p24	+	–	75%
BA-3	G2b	gp100/CALLA	+	–	70%

[a] Percentage of cases which were positive

Table 2. Monoclonal antibodies BA-1, BA-2, BA-3, and complement do not inhibit the growth of human hematopoietic stem cells[a]

Treatment[b]	CFU-GEMM	CFU-GM	BFU-E	CFU-E
Bone marrow alone	10.7±1.5	28.5±4	42.8±5.9	138±17
Bone marrow + complement	9.9±1.3	30.0±3.5	35 ±4.2	69± 9
Bone marrow + BA-1, BA-2, BA-3, and complement	11.3±1.2	33 ±2.1	40.5±3.7	85±14

[a] The four types of human hematopoietic stem cell colonies (CFU-GEMM, CFU-GM, BFU-E, CFU-E) were assayed in vitro using standard techniques. Data are expressed as the number of colonies per 10^5 cells plated from a single, normal bone marrow donor

[b] All three bone marrow groups were incubated for 1 h at 37° on a rocker platform prior to plating for the individual stem cell assays. BA-1, BA-2, and BA-3 were used at a final dilution of 1 : 100. Rabbit complement was used at a final dilution of 1 : 6

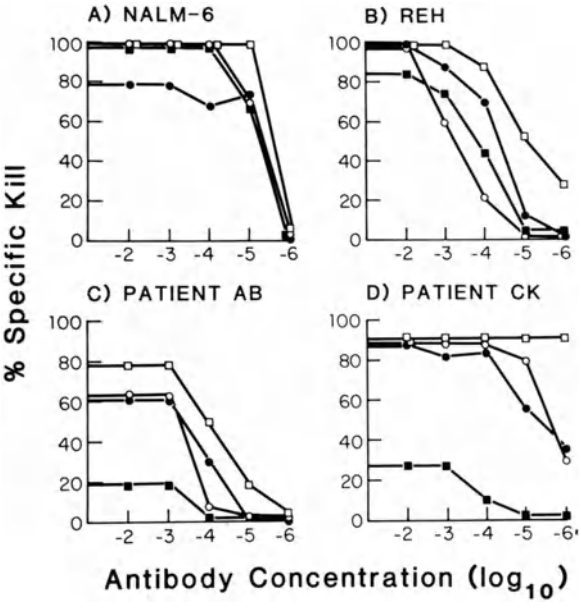

Fig. 1. Comparison of individual antibodies versus the antibody cocktail for cytolysis of leukemic cells. ●, BA-1 + complement; ○, BA-2 + complement; ■, BA-3 + complement; □, BA-1, BA-2, BA-3 + complement

Prior to utilizing BA-1, BA-2, and BA-3 for the ex vivo elimination of leukemic cells in autologous bone marrow transplantation, a series of experiments were undertaken to define the optimal conditions for their in vitro use.

As mentioned above, we had previously shown that when used individually with rabbit complement, neither BA-1, BA-2, nor BA-3 inhibited bone marrow stem cell growth in vitro. Thus, the first question we asked was whether BA-1, BA-2, and BA-3 *together* (antibody cocktail) inhibited stem cell growth. The results of one such experiment are shown in Table 2. The results clearly indicate that incubation of normal bone marrow with antibody cocktail plus rabbit complement did not inhibit the growth of CFU-GEMM, CFU-GM, and BFU-E. There was some suppression of CFU-E, but this also occurred with complement alone, suggesting that the effect was not antibody dependent.

We then asked whether antibody cocktail plus rabbit complement was more effective than any single antibody at lysing target cells. The results obtained with four individual target cells are shown in Fig. 1. It is apparent that the antibody cocktail plus rabbit complement effectively lysed all four targets. These targets included the cell lines NALM-6 and REH, and leukemic cells from two newly diagnosed ALL patients, AB and CK. Particularly noteworthy were the results obtained with leukemic cells from patient AB. Bone marrow from this patient had approximately 80% malignant cells by morphology. Immunologic phenotyping demonstrated 60% BA-1^+ cells, 60% BA-2^+ cells, and 20% BA-3^+ cells. The results show that treatment with the antibody cocktail lysed more cells than treatment with any single antibody. Most importantly, treatment with the antibody cocktail appeared to lyse all the morphologically malignant cells. Also, in no instance did the antibody cocktail result in less effective killing than any single antibody, thereby eliminating the possibility that use of the antibody cocktail would lead to steric hindrance of antibody binding at the cell surface. The data presented in Fig. 1 are based on analysis of cytotoxicity by trypan blue exclusion. Similar results were obtained using ^{51}Cr-release.

The next series of experiments were designed to determine if the BA-1, BA-2, and BA-3 antibody cocktail could effectively kill small numbers of leukemic cells in normal bone marrow. Table 3 summarizes the results of four preliminary experiments using three different leukemic cell lines. These experiments were conducted by incubating bone marrow for 1 h at 37 °C. In

data not shown, these conditions were found to be optimal for lysis of leukemic cells. The antibody cocktail was highly effective at eliminating ^{51}Cr-labeled leukemic cells in the presence of a 10-fold, 100-fold, or 1000-fold excess of normal bone marrow cells. In some instances we were not able to effectively lyse all ^{51}Cr-labeled leukemic cells (experiments 1 and 2). The reasons for this are not immediately apparent but may be explainable on the basis of variability inherent within the ^{51}Cr-release assay.

Table 3. In vitro cytodestruction of leukemic cells in human bone marrow using BA-1, BA-2, and BA-3, and complement

	% leukemic cells		
Cell line[a]	10	1	0.1
REH			
Exp. No. 1	100[b]	100	94.6
Exp. No. 2	99.8	100	97.9
NALM-6			
Exp. No. 1	100	100	100
KOPN-1			
Exp. No. 1	100	100	100

[a] REH = 99% BA-1$^+$, 50% BA-2$^+$ (weak), 95% BA-3$^+$; NALM-6 = 75% BA-1$^+$, 99% BA-2$^+$, 98% BA-3$^+$; KOPN-1 = 60% BA-1$^+$, 50% BA-2$^+$, 99% BA-3$^+$

[b] Values expressed as % specific ^{51}Cr-release using BA-1, 2, 3, and C

The experiments reported herein represent our initial efforts at optimizing the conditions necessary for the ex vivo elimination of residual leukemic cells with the BA-1, BA-2, and BA-3 antibody cocktail. Our decision to use all three antibodies is based on their binding to non-T ALL, and their inability to inhibit the in vitro growth of bone marrow stem cells in the presence of rabbit complement. Furthermore, we do not currently know whether the cell surface molecules recognized by BA-1, BA-2, and BA-3 are expressed on the *clonogenic* cell in any given case of ALL. An in vitro assay for the clonogenic ALL cell has recently been developed [17] and should assist in addressing the issue of the surface phenotype. For these reasons, we feel that the three antibodies together may be more effective than any single antibody alone.

We have recently initiated a phase I clinical trial to test the efficacy of the BA-1, BA-2, BA-3 antibody cocktail plus complement for the ex vivo elimination of leukemic cells in autologous bone marrow transplantation. Similar trials are underway using the J-5 anti-CALLA monoclonal antibody [18] and the anti-Leu 1 monoclonal antibody [19]. Hopefully, these trials will result in improved therapeutic results in patients who are currently not successfully treated with conventional chemotherapy.

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In Vitro Treatment with Monoclonal Antibody Prior to Autologous Bone Marrow Transplantation in Acute Lymphoblastic Leukemia *

J. Ritz, S. E. Sallan, R. C. Bast, Jr., J. M. Lipton, D. G. Nathan, and S. F. Schlossman

A. Introduction

Monoclonal antibodies which are specific for surface antigens of leukemic cells have become useful diagnostic reagents and have been used to dissect the heterogeneity of leukemia in man [1, 2]. In addition, it is apparent that large quantities of homogeneous antibody which primarily react with leukemic cells may become useful therapeutic reagents. Previous trials of serotherapy with various monoclonal antibodies in patients with multiply relapsed acute lymphoblastic leukemia (ALL) or lymphoma have demonstrated that intravenously administered antibody can rapidly bind to tumor cells in peripheral blood and bone marrow and that relatively large numbers of malignant cells can be eliminated in vivo [3–6]. In one patient with B-cell lymphoma, a complete remission was achieved following intravenous infusion of monoclonal anti-idiotype antibody [7]. In general, however, these studies have not produced clinically significant responses and have clearly identified several specific factors such as presence of serum-blocking factors, antigenic modulation, and inefficiency of natural effector mechanisms, which limit the therapeutic activity of monoclonal antibody in vivo (reviewed in [8]).

One approach which circumvents several of the obstacles to effective serotherapy in vivo is the utilization of monoclonal antibody in vitro. Thus, in a controlled in vitro environment, extracellular blocking factors can be removed, incubation with monoclonal antibody at 4 °C can effectively inhibit antigenic modulation, and multiple treatments with heterologous complement can be used to ensure the lysis of all tumor cells. In addition, potential cross reactivity of monoclonal antibodies with non-hematopoietic tissues can be avoided. A previous report has presented our preliminary experience with the use of the J5 monoclonal antibody and rabbit complement to treat bone marrow in vitro to remove residual leukemic cells prior to autologous transplantation [9]. This report summarizes the current results of this clinical study.

B. Methods

I. J5 Monoclonal Antibody

The method for generation and characterization of J5 monoclonal antibody specific for the common acute lymphoblastic leukemia antigen (CALLA) has been described previously [10]. J5 antibody (murine IgG2A) is reactive with leukemic cells from 80% of patients with non-T cell ALL and 40% of patients with chronic myelocytic leukemia in blast crisis. In addition, lymphoma cells from almost all patients with B-cell nodular poorly differentiated lymphocytic lymphoma and Burkitt's lymphoma, and 45% of patients with T-cell lymphoblastic lymphoma, are reactive with

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J5 antibody [11]. Within normal bone marrow, approximately 1% of cells also express CALLA, but previous studies have shown that these normal CALLA-positive cells are not myeloid precursor cells (CFU-C, BFU-E, CFU-E, and CFU-G/E) [12].

More recent studies have indicated that CALLA is expressed during early lymphoid cell differentiation, but it appears that the earliest lymphoid stem cells do not express this antigen [13]. In addition to hematopoietic cells, it has been demonstrated that J5 antibody is reactive with various nonhematopoietic tissues including cells from renal glomerulus and proximal tubules [14]. Recently, it has also been found that J5 antibody is reactive with cultured fibroblasts from normal bone marrow (J. Ritz, unpublished observation) as well as cell lines established from various solid tumors (H. Lazarus, personal communication). These findings are of particular importance for the therapeutic application of CALLA-specific antibodies since these normal cells would also be potential targets for antibody-directed therapy.

Our method for obtaining large quantities of purified J5 monoclonal antibody and our method for in vitro treatment of bone marrow have been previously described [9, 15, 16]. Briefly, J5 antibody was obtained aseptically from ascitic fluid of Balb/c mice that had been primed with pristane followed by intraperitoneal inoculation of J5 hybridoma cells. Bone marrow was harvested from anterior and posterior iliac crests under general anesthesia, and mononuclear cells were isolated using discontinuous Ficoll-Hypaque density gradients. Bone marrow cells were then treated three times with J5 antibody and rabbit complement and cryopreserved in the vapor phase of liquid nitrogen in media containing 10% DMSO and 90% autologous seurm. Prior to infusion, cryopreserved marrow was rapidly thawed and cells were diluted in medium which contained DNAase to prevent clumping.

C. Results

I. Clinical Protocol

All patients with ALL who had relapsed following standard chemotherapy and whose leukemic cells expressed the common ALL antigen (CALLA) were considered eligible for the protocol that is outlined in Fig. 1. Patients who had normal identical twins or histocompatible siblings were ineligible for this study and received either syngeneic or allogeneic bone marrow transplantation. In addition, patients in whom a complete remission could not be induced with chemotherapy alone were excluded.

Following induction of second or subsequent remission, patients received intensive chemotherapy with the following agents: VM-26, cytosine arabinoside (araC), and *L*-asparaginase (Fig. 1). CNS repropylaxis with intrathecal araC and hydrocortisone was also adminstered at that time. After recovery from intensification, patients underwent bone marrow harvest under general anesthesia. Mononuclear cells were isolated and treated three times with J5 antibody and rabbit complement prior to cryopreservation. A separate aliquot of marrow was also cryopreserved without antibody treatment. These cells constituted a "back-up marrow" which could be used in the event that antibody-treated marrow failed to engraft but was not used in any of our patients. In patients 1, 2, and 3, "back-up marrow" was harvested separately just prior to intensification and cryopreserved without antibody treatment.

One day after marrow harvest, patients began receiving ablative treatment consisting of VM-26, araC, cyclophosphamide, and total body irradiation (TBI) (Fig. 1). Approximately 12 h after TBI, cryopreserved marrow which had previously been treated in vitro was rapidly thawed and reinfused through a central venous catheter. Patients did not receive any additional chemotherapy.

Thus far, six patients have been treated under this protocol and have been followed for more than 4 months. The clinical history of these patients and their current status is summarized in Table 1. Patient 1 had relapsed in bone marrow 20 months after elective cessation of therapy and now continues in unmaintained remission 20 months after autologous transplantation. Patient 2 had relapsed in both testes 2 months after completion of chemotherapy.

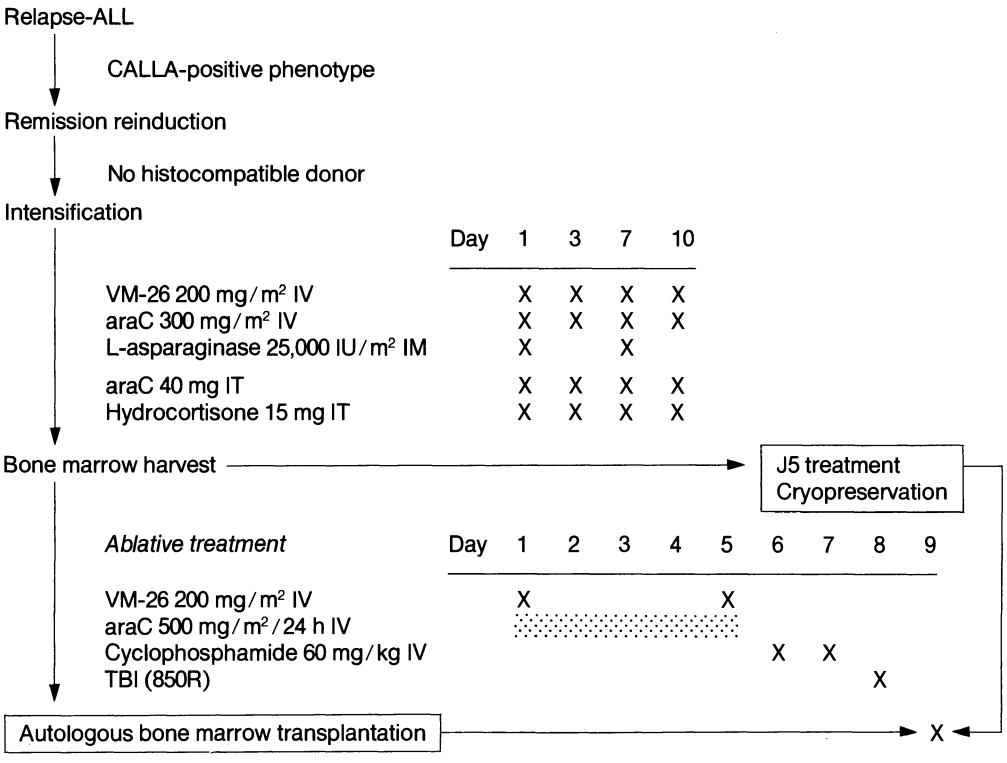

Fig. 1. Clinical protocol for autologous transplantation with J5 antibody and complement-treated bone marrow

Bone marrow at that time contained 7% lymphoblasts. He continues in unmaintained remission 18 months after autologous bone marrow transplantation. Patient 3 first relapsed in the CNS while receiving systemic chemotherapy and later relapsed in the bone marrow as well. A second bone marrow remission was difficult to achieve and was only attained after 4 months of intensive chemotherapy. He relapsed with CALLA-positive lymphoblasts 7 weeks after transplantation. Patient 4 relapsed in the bone marrow 3 months after initial diagnosis. He tolerated the ablative regimen well but subsequently developed interstitial pneumonitis, which was probably secondary to cytomegalovirus infection and expired 3 months after transplantation. Pneumonitis was also complicated by intrapulmonary hemorrhage secondary to persis-

Table 1. Clinical characteristics of patients treated with autologous bone marrow transplantation

Patient	Age	Sex	Initial WBC/mm³	Duration of 1st remission	Relapse site	Postautologous transplant status
1	10	M	2,000	50 months	BM	CR 20 months
2	5	M	150,000	32 months	Testes	CR 18 months
3	3	M	22,000	15 months	BM/CNS	Rel 7 weeks
4	4	M	31,000	2 months	BM	Exp 3 months in remission
5	14	M	98,000	29 months	BM/testes	5 months
6	11	M	5,900	7 years	BM/CNS/ testes	4 months

tent thrombocytopenia. At autopsy, there was no evidence of leukemic relapse. Patient 5 was transplanted in third remission. He first relapsed in the bone marrow 30 months after initial diagnosis. and subsequently continued on chemotherapy for an additional 4 years until therapy was electively stopped. Testicular relapse with CALLA-positive cells occurred 8 months later. Morphologic examination of bone marrow at this time demonstrated 3% blasts but immunofluorescence analysis of purified mononuclear cells demonstrated 19% CALLA-positive cells. He was subsequently entered onto our protocol and continues in remission 5 months after transplantation. Patient 6 received chemotherapy for 5 years after initial diagnosis but relapsed simultaneously in the bone marrow, CNS, and testes 2 years after elective cessation of therapy. Following reinduction of a second complete remission, he received the intensification and ablative therapy outlined in Fig. 1. He continues in remission 4 months after autologous transplantation.

II. Hematopoietic Reconstitution

Hematopoietic engraftment in six patients following autologous transplantation with J5-treated bone marrow is summarized in Table 2. In patient 1, the first evidence of marrow engraftment was seen 11 days after marrow infusion, and subsequent recovery of granulocytes, reticulocytes, and platelets occurred promptly. In patient 2, the first evidence of marrow engraftment was seen 9 days after transplant, but subsequent hematopoietic recovery occurred slowly. Although complete recovery did eventually occur, severe thrombocytopenia persisted for 3 months.

In patient 3, hematopoietic reconstitution proceeded gradually after marrow infusion, but bone marrow relapse became evident before complete recovery of peripheral counts had occurred. Seven weeks posttransplant, bone marrow aspirate demonstrated engraftment of granulocytic, erythroid, and megakaryocytic precursors but also contained approximately 40% CALLA-positive lymphoblasts. Patient 4 exhibited prompt recovery of granulocytes but reconstitution of both platelets and reticulocytes was much slower. Although megakryocytes were present in bone marrow aspirates and at autopsy, circulating platelet counts remained $< 20{,}000\ mm^3$.

Patients 5 and 6 have been followed for relatively short periods, but hematopoietic recovery in both of these patients appears to be comparable to that seen in previous patients.

III. Immunologic Reconstitution

The appearance of B cells in peripheral blood and bone marrow was detected by reactivity with monoclonal antibody B1 which identifies a unique antigen expressed by normal B cells [17]. In patients 1 and 2, serum immunoglobulin levels gradually increased following the appearance of B1-positive cells.

In all six patients, T-lymphocytes were the first cells to engraft following transplantation. These cells expressed T3, T10 [18], and Ia antigens [19]. Although T cells from

Table 2. Hematopoietic recovery following autologous bone marrow transplantation

	Day S/P transplant					
Patient	1	2	3	4	5	6
Hematologic						
1st granulocyte	11	9	9	11	10	10
Granulocytes $>1000/mm^3$	49	78	54	56	43	63
Platelets $>25{,}000/mm^3$	22	92	–	–	62	49
Reticulocytes $>0.9\%$	22	36	25	69	45	47
Immunologic						
B cells (B1+)	63	128	–	61	45	64
T cells (T3+, T10+, Ia+)	11	9	10	12	9	11

peripheral blood normally express T3 antigen, both T10 and Ia antigens are normally expressed only after cell activation [20, 21]. Both T4 cells (T-inducer phenotype) and T8 cells (T-suppressor phenotype) were present, but the relative percentage of these cells in peripheral blood varied during engraftment. In almost all patients, the T4/T8 ratio of circulating T cells was abnormally low. In patients who initially had normal percentages of T4-positive cells (patients 1 and 5), peripheral T cells also later became predominantly T8 positive. The number of T cells which were Ia positive gradually decreased during the first 4 months following engraftment. Expression of T10 antigen also gradually decreased following engraftment but persisted much longer. At no time during engraftment was T6 antigen expressed by peripheral blood cells.

D. Discussion

Autologous bone marrow transplantation has previously been used in patients with various malignant diseases in an effort to circumvent marrow toxicity and to allow the administration of otherwise lethal doses of chemotherapy. Unfortunately, in most patients with solid tumors, higher doses of chemotherapy have not resulted in more effective eradication of malignant cells. In contrast, it has been demonstrated that leukemia and lymphoma cells can be eradicated with intensive chemotherapy and total body irradiation (TBI) even when these tumors are resistant to conventional doses of chemotherapy. This has led to the successful treatment of acute leukemia with ablative chemotherapy and TBI in conjunction with bone marrow transplantation from identical twins or allogeneic histocompatible siblings [22–25]. Unfortunately, the utilization of ablative therapy in leukemia is restricted to approximately 40% of patients who have normal histocompatible marrow donors, and autologous marrow transplantation is limited by the fact that residual leukemia is present in the patient's marrow, even during complete remission, Previous studies have attempted to circumvent this problem through the use of physical separation techniques [26] or treatment with conventional rabbit heteroantisera [27, 28] to eliminate leukemic cells prior to autologous transplantation. High-titer monoclonal antibodies which activate complement and specifically react with leukemic cells and not with hematopoietic stem cells are potentially very useful reagents which can be used to eliminate small numbers of leukemic cells in the presence of a large excess of normal marrow. The utilization of these reagents in vitro may therefore allow the application of autologous bone marrow transplantation to patients who do not otherwise have histocompatible donors.

In the present study, six patients with relapsed ALL received ablative therapy with VM-26, araC, cyclophosphamide, and TBI followed by infusion of autologous remission bone marrow which had been treated in vitro with J5 antibody and rabbit complement to remove residual leukemic cells. Hematopoietic engraftment with J5-treated bone marrow occurred in all six patients. Reconstitution of B cells and immunoglobulin production occurred after reconstitution of myeloid cells. Since all of our patients have engrafted with J5 antibody-treated bone marrow and two patients have been in unmaintained remission for more than 18 months, our study suggests that this approach may be a feasible alternative to conventional chemotherapy in patients with relapsed ALL. Treatment of additional patients and longer follow-up periods will be necessary to determine if in vitro antibody treatment is a clinically effective therapeutic modality.

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Immunological Classification of Acute Lymphatic Leukemia

P.-M. Chen and C.-K. Ho

Lymphocytes have conventionally been classified as T, B, and null cells, based on their characteristic surface membrane markers. Such membrane features can be revealed by rosette assays and by immunofluorescence using heterologous and/or monoclonal antibodies. Studies of these characteristics can provide important insight into the differentiation scheme of normal lymphocytes and can be applied to the classification of leukemic cells. This is a preliminary report on the studies of immunological markers of leukemic cells from acute lymphocytic leukemia (ALL) patients in an attempt to classify these cells into specific subtypes and to correlate these membrane features with prognostic and pathogenic characteristics.

Leukemic cells from 54 patients could be classified into eight subtypes, based on immunological phenotypic criteria as listed in Table 1. The percent distribution of the different subtypes were: null (0%), Ia (17%), cALL (52%), pre-B (4%), B (4%), defective B (8%), pre-T (4%), early T (8%), and T1 (5%). There were no apparent differences among the cALL, Ia, and the various types of B-ALL in clinical findings and in general, Ia, cALL, pre-B, and defective B patients had higher remission rates and better responses to chemotherapy while the others had poorer prognosis and higher incidences of drug resistance as reported elsewhere [2].

There has been some controversy over the classification criteria of our non-T non-B cells and pre-T ALL. Our standard criteria for non-T non-B (null, Ia, cALL) cells are those proposed by Chessells et al. [3] and Brouet and Seligmann [1], and our definition for pre-T cells is based on reac-

Table 1. Identification of ALL leukemic cells by surface marker analysis

Type	Subtype	Ia	cALL	OKT9	10	11A	S33	WT1	SIg[a]	CmIg	E(h/c)	EA/EAC
				Monoclonal antibodies								
Non-T, non-B	Null	–	–	–	–	–	–	–	–	–	–	–
	Ia	+	–	–	+/–	–	–	–	–	–	–	–
	cALL	+	+	–	+/–	–	–	–	–	–	–	–
B	Pre-B	+	+/–	–	–	–	–	–	–	+	–	–
	Def B	+	–	–	–	–	–	–	–	–	–	+
	B1	+	–	–	–	–	–	–	+	–	–	+
T	Pre-T	+/–	+/–	–	+	+/–	+	+	–	–	–	–
	Early T	+/–	–	+	+	+/–	+/–	+	–	–	–	–
	T1	–	–	–	+	+	+	+	–	–	+	–

[a] SIg, surface immunoglobulin; CmIg, cytoplasmic immunoglobulin; E(h/c), hot/cold E rosette; EA/EAC, EA and EAC rosettes

Table 2. Percentage of T-ALL leukemic cells reactive to a selected panel of monoclonal antibodies and other assays

Case No.	Subtype	E(h/c)	OKT3	9	10	11A	S33	WT1	SIg	cALL	Ia	Thymic mass
			Monoclonal antibodies									
1[a]	pre-T	0/5	7	0	90	92	90	99	0	0	94	–
2	pre-T	0/1	0	0	42	3	63	95	0	32	0	+
3	early T	0/2	1	78	99	99	99	99	0	0	0	–
4	early T	0/3	3	45	90	3	99	99	0	9	0	+
5	early T	0/3	3	79	90	7	7	99	0	0	90	+
6	early T	0/2	2	72	90	NT	NT	NT	0	0	0	+
7	T1	40/96	NT	NT	NT	NT	NT	NT	0	0	0	+
8	T1	20/58	20	NT	NT	32	23	NT	0	2	8	+
9	T1	31/81	81	NT	NT	18	33	23	1	7	4	+

NT, not tested

[a] Based on our previous classification criteria (see text for description), cases 1 and 5 were diagnosed as Ia subtype; case 2 was cALL and cases 3, 4, and 6 were null subtype

tivity to OKT10 but not to other OKT monoclonal antibodies as reported by Reinherz et al. [4]. However, further analysis with additional monoclonal antibodies OKT11A, S33, and WT1 have shown that (1) at least some of our non-T non-B cases can be reclassified into the T-cell category and (2) pre-T and early T subtypes of ALL can be better defined with the aid of OKT11A, S33, and WT1. Together with OKT9/10, this panel of monoclonal antibodies defines pre-T ALL as $OKT9^{-}/10^{+}$ and $OKT11A^{\pm}/S33^{+}/WT1^{+}$ while early T-ALL can be distinguished as $OKT9^{+}/10^{+}$ and $OKT11A^{\pm}/S33^{\pm}/WT1^{+}$ (Table 2). Based on these new criteria, all of our null-ALL, one of cALL, and two of our Ia-ALL have been reclassified as either pre-T or early T-ALL as shown in Table 2. Our data thus suggest that OKT11A, S33, and WT1 monoclonal antibodies should be included in the OKT regiment for the phenotypic studies of T and stem (non-T non-B) cell subtypes. Furthermore, the characteristic markers of the "null" cell subtype should be reexamined or the term "null cell" be abandoned.

In conclusion, we have been able to classify 54 acute lymphocytic leukemic cases into eight subtypes based on surface marker analysis and a panel of monoclonal antibodies. Our preliminary data have casted some doubts on the immunological classification criteria of some subtypes of ALL. Furthermore, we have shown that the use of monoclonal antibodies OKT11A, S33, and WT1 may be very useful in defining pre-T and early T subtypes of ALL and in differentiating between stem cells and pre-T cells. Attempts are in progress to further substantiate these suggestions.

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Multimarker Analysis of Childhood Acute Lymphoblastic Leukemia (ALL): Heterogeneity of Cellular Phenotypes and Clinical Relevance of Immunological Defined ALL Subclasses*

I. Thoene and H. Kabisch

A. Introduction

The comparative study of normal and leukemic cells by means of immunological phenotyping has provided insights into the origin of the malignant cell clones and the putative "target" cells of malignant transformation. The similarity found between normal and leukemic cell phenotypes suggests that leukemic blasts represent lymphoid cells which are arrested at a given stage along the individual differentiation pathway [4].

In the majority of childhood ALL (ca. 70%) the blasts exhibit the composite cellular phenotype of normal lymphoid bone marrow precursor cells: they are positive for the "common" ALL antigen (CALLA) and the HLA-DR/Ia antigen complex and express the nuclear enzyme terminal transferase (TdT) [5]. In approximately 20% of "common" ALL cases the cells additionally show small amounts of cytoplasmic mu chains [13], thus reflecting the phenotype of normal bone marrow pre-B cells [7]. Therefore during childhood this cell type probably represents a main candidate for leukemic transformation. Other childhood ALL subclasses are T-ALL (ca. 20%) and the rare B-ALL (1%–2%). In 10%–20% of ALL the origin of the blasts is unknown (U-ALL). It has been documented in several studies that the biological heterogeneity of leukemic cells is linked to a remarkable clinical heterogeneity. Thus T-ALL is usually associated with a higher initial blast count than "common" ALL. A possible explanation for the latter finding could be a different site of proliferation of malignant clonogenic cells (bone marrow in "common" ALL versus thymus in T-ALL). Prognostically the immunological phenotype is not an independent parameter since it is linked to other important clinical features (e.g., tumor load). However, the enormous importance of leukemic cell phenotyping for our understanding of the biology of malignant cells, for the improvement of diagnosis, and for a better definition of patients with high-, standard-, or low-risk disease is undoubted.

In the following we report on our experiences with leukemic cell phenotyping. The main aims of this study were (a) to investigate the heterogeneity of ALL subclasses with special emphasis on the U-ALL subclass and (b) to analyze the prognostic value of ALL subtyping.

B. Materials and Methods

Leukemic cells were derived from heparinized bone marrow and blood samples of children with previously untreated ALL. All patients have been treated in a cooperative study (COALL 80) according to the Hamburger protocol ALL V/79, which is a less aggressive modification of the West Berlin Study Programm BFM 76/79 [14]. In all cases diagnosis had been confirmed by conventional morphological and cytochemical methods. Immunological multimarker analyses was performed using the following reagents: an extensively absorbed rabbit antiserum against non-B –

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non-T ALL cells was used for the detection of the "common" ALL antigen. Later in the study this serum was replaced by monoclonal antibodies J-5 [11] and VIL-A 1 [10]. A cytotoxic heteroantiserum against T-cell antigen (anti-HUTLA) was prepared in rabbits which were immunized with thymic cells from children undergoing cardiac surgery. After absorption with normal and leukemic B cells this antiserum exclusively reacted with thymocytes and T-cell leukemias. Membrane immunoglobulin (Ig) was detected with commercial FITC-labelled polyvalent $F(ab)_2$ rabbit anti-human Ig antiserum.

For TdT detection an affinity-purified rabbit anti-calf TdT antiserum was used ([2]; BRL, United Kingdom). Monoclonal antibody DA2 recognizes a framework structure of the HLA-DR/Ia antigen complex [3] whereas monoclonal antibody Y 29/55 reacts with an epitope restricted to cells of B lineage [6].

Monoclonal antibodies BA1 [1] and BA2 [8] detected determinants highly specific for cells of lymphoid origin. Furthermore mononclonal antibodies from the OKT series (ORTHO, New Jersey) namely OKT3, OKT4, OKT6, and OKT8, were used. The E rosette assay was performed with neuraminidase-treated sheep red blood cells according to standard procedures. Binding of peanut (*Arachis hypogaea*) lectin was studied using TRITC-labelled affinity-purified peanut agglutinin (MILES) [12].

C. Results and Discussion

I. Heterogeneity of Phenotypes

In a prospective clinical study between 1979 and 1981 the blast from 54 children with previously untreated ALL were classified by immunological marker analysis. Table 1 shows the marker profile of the four ALL subclasses detected in this study. In 34 cases the blasts showed the typical phenotype of "common" ALL cells, i.e., CALLA+Ia+TdT+. In three cases a considerable number of cells (over 30%) were positive for B-cell specific markers: Ig or Y29/55. These findings indicate a partial differentiation of the blasts along the B-cell differentiation pathway and can be explained by the fact that ca. 20% of "common" ALL are pre-B cell leukemias [13]. The absence of T-cell markers in "common" ALL and recent results from Ig gene rearrangement studies [9] in ALL cells may suggest that a higher proportion of "common" ALL is derived from transformed pre-B cells. In our study ten cases could be identified as T-cell malignancies (seven T-ALL, three T-NHL). In every case the blasts were HUTLA positive and HLA-DR/Ia negative, whereas only one case was CALLA positive. Using the E rosette assay, anti-TdT, and the monoclonal antibodies of the OKT series a remarkable heterogeneity within this ALL subclass was detectable; e.g., in five of ten cases the T blasts did not bind sheep red blood cells; one of seven cases was TdT negative, and only three of eight reacted with OKT antibodies. The one patient with B-ALL showed blasts with monotypic surface Ig composed of mu and kappa chains.

Nine patients had blasts which were unclassifiable with the marker panel used in this study. However, as is shown in Table 1, the so called acute "unclassifiable" leukemias U-ALL do not represent a homogeneous subclass. The exact delineation of U-ALL cells is of utmost clinical importance in that some U-ALL may be of myeloic origin and need a different treatment. For this reason we used among other markers the monoclonal antibodies BA1 and BA2 and a plant lectin – the peanut agglutinin – in an attempt to further dissect this subclass.The results are shown in Table 2. Per definitionem all cases are negative for B- and T-cell markers and CALLA.

The following different U-ALL phenotypes could be identified: (1) Ia−TdT−; (2) Ia+TdT−; (3) Ia+TdT−BA1−BA2+; (4) Ia+BA1+BA2+; (5) Ia+TdT−BA1+BA2+; and (6) Ia−TdT−BA1−BA2−. Two out of five cases were positive for peanut lectin receptors. One of these cases (F.H.) was negative for all other markers. Of special value in the subclassification of U-ALL are the antibodies BA1 and BA2 in that these antibodies recognize determinants which are highly restricted to lymphoid lineage cells. However, the many questions concerning the origin and clinical features of U-ALL subclasses can only be answered in a larger study.

n (%)	"Common" ALL	T-ALL/NHL	B-ALL	U-ALL
	34 (62.9)	10 (18.5)	1 (1.9)	9 (16.7)
Anti-CALLA	34/34	1/10	0/1	0/9
Anti-TdT	20/21	6/7	0/1	3/9
Anti-Ia	34/34	0/10	1/1	5/9
Anti-Ig	2/34	0/10	1/1	0/9
Y29/55	1/13	0/8	1/1	0/7
Anti-HuTLA	0/34	10/10	0/1	0/9
SRBC	0/34	5/10	0/1	0/5
OKT3	0/21	0/7	0/1	0/5
OKT4	0/21	3/8	n.t.	0/5
OKT6	0/21	3/8	n.t.	0/5
OKT8	0/21	3/8	n.t.	0/5

Table 1. Reactivity of ALL blasts of 54 children with different markers

II. All Subclasses and Response to Treatment

Table 3 shows the clinical data in relation to the immunological ALL subclasses of the 54 children included in the prospective study. The most striking differences between the different subclasses are (a) a higher mean age in U-ALL and T-ALL/NHL, (b) a predominance of males in T-ALL/NHL and U-ALL, (c) a higher mean platelet count in T-ALL/NHL, and (d) a lower mean WBC in "common" ALL. The two patients with mediastinal mass both had T-NHL. High-risk and low-risk patients were defined on the basis of initial WBC (high-risk WBC over 25×10^9/liter). Children with high-risk disease received a reinforced reinduction therapy. As shown in Table 4 relapses occurred in all ALL subclasses. With respect to the patient fractions in first CCR a life table analysis revealed no significant difference between the only immunologically defined ALL subclasses. A remarkablely worse outcome showed patients with T-NHL but not those with T-ALL. These data confirm the finding of the BFM study group that with intensified treatment programs the T-ALL shows no worse prognosis than non-T ALL. However, on the basis of the immunological subtype and initial WBC count we were

Table 2. Reactivity of U-ALL cells with a selected marker panel

Patient	Age (years)	Sex	CALLA	SmIg	HuTLA	Ia	TdT	BA1	BA2	PNA
1. S.H.	16	m	–	–	–					
2. E.R.	13	m	–	–	–					
3. G.A.	8	m	–	–	–					
4. G.C.	10	m	–	–	–					
5. H.K.	18	m	–	–	–	+				
6. R.M.	10	f	–	–	–	+		–		
7. B.S.	11	f	–	–	–	+	+			–
8. C.C.	1.5	f	–	–	–	+	+			
9. S.P.	19	f	–	–	–	+	+			
10. H.R.	11	f	–	–	–	–				
11. V.G.	8	m	–	–	–	+	–			(+)
12. H.M.	1	m	–	–	–	+	+	–	+	–
13. C.G.	12	m	–	–	–	+	–	+	+	–
14. R.R.	9	m	–	–	–	+		+	+	
15. H.F.	7	m	–	–	–	–	–	–	–	+
16. Y.B.	12	m	–	–	–	+				

n	"Common" ALL	T-ALL/ NHL	B-ALL	U-ALL
	34	10	1	9
Age (mean)	4.5	6	11	9
< 2 years	1	1		1
> 10 years	4	4		4
Sex m/f	16/18	9/1	0/1	6/3
Platelet count (mean) ($\times 10^9$/l)	32	111	134	60
HK (1/1)	21	26.5	38	28
WBC ($\times 10^9$/l) (mean)	5.9	10.2	5.8	10.2
<25	29	6	1	5
>25	5	4		4
CNS involvement at diagnosis	2	2	0	0
Mediastinal mass at diagnosis	0	2	0	0

Table 3. Clinical details of immunologically defined ALL subclasses

ALL subclass	Relapse frequency (cumulative proportion in first CCR)		
	High-risk	Low-risk	Total
"Common" ALL	2/ 5 (0.6)	1/29 (0.97)	3/34 (0.91)
T-ALL	0/ 4 (1.0)	1/ 3 (0.67)	1/ 7 (0.86)
T-NHL			2/ 3 (0.33)
B-ALL		1/ 1 (0.0)	1/ 1 (0.0)
U-ALL	1/ 4 (0.75)	1/ 5 (0.80)	2/ 9 (0.78)
	3/13 (0.77)	4/38 (0.89)	9/54 (0.84)

Table 4. Immunologically defined ALL subtypes and response to treatment. Modified from Winkler et al. [15]

able to define a patient fraction with a cumulative proportion in first complete clinical remission CCR of 0.97: these patients had "common" ALL and less than 25×10^9 WBC/liter. The cumulative proportion in first CCR of patients with "common" ALL and over 25×10^9 WBC/liter, T-ALL/NHL, B-ALL, or U-ALL taken as one group is, at 0.68, significantly lower ($P < 0.05$). From the results of this study it is concluded that for further approaches to individualized therapy programs (i.e., reduction of ineffective toxicity) immunological phenotyping is a powerful tool for the identification of patients with high- and low-risk disease.

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Morphological and Cytochemical Features of Adult T-Cell Lymphoma-Leukaemia

A. D. Crockard, M. O'Brien, D. Robinson, J. Tavares de Castro, E. Matutes, and D. Catovsky

A. Introduction

T-cell malignancies may be recognised in several distinctive forms: T-lymphoblastic leukaemia and lymphoblastic lymphoma which are proliferations of immature T cells, and a heterogeneous group of proliferative disorders with a mature T-cell phenotype, T-chronic lymphocytic leukaemia, T-prolymphocytic leukaemia and the cutaneous T-cell lymphomas [1]. Within this spectrum of diseases, a distinctive T-cell leukaemia-lymphoma affecting black adults of West Indian/Caribbean origin has been recognised by our group [2] and designated adult T-cell leukaemia-lymphoma (ATLL). The salient features of the disease include its occurrence in black West Indians, the presence of high titres of antibody against the p 24 structural core protein of human T-cell leukaemia-lymphoma virus (HTLV), severe hypercalcaemia without bone lesions, lymphadenopathy, high WBC and short survival. Immunologically the malignant cells are of mature post-thymic phenotype (E+, TdT−, OKT3+, OKT6−), and in those cases tested with OKT4 and OKT8 monoclonal antibodies, of helper/inducer phenotype (OKT4+, OKT8−).

In this communication we describe the light (LM) and electron microscopic (EM) morphological and cytochemical features of the malignant T cells in this condition.

B. Materials and Methods

Peripheral blood (PB) and/or bone marrow (BM) films from six adult T-cell lymphoma-leukaemia patients were stained with May-Grünwald Giemsa and examined under LM. For EM analysis, PB and BM cells were fixed in 3% glutaraldehyde and embedded in Araldite. Ultrathin sections stained with uranyl acetate and lead citrate were then viewed through a Zeiss 10 electron microscope. The following cytochemical reactions: acid phosphatase, α-naphthyl acetate esterase (ANAE), β-glucuronidase, β-glucosaminidase and periodic acid schiff (PAS) were performed on PB and BM films, as described previously [3].

C. Results

I. LM Morphology

Atypical lymphoid cells from PB and BM displayed marked variation in size, maturity and nuclear outline. A high nuclear/cytoplasmic ratio was a consistent finding in the neoplastic cells irrespective of size, which varied from that of a small lymphocyte to that of a large blast. Most cells appeared mature (chromatin condensed), although small populations of cells of blastic appearance (prominent nucleoli) were also identified. A characteristic feature of the malignant cells was that of nuclear irregularity (Fig. 1). Folded, notched or lobulated nuclei, similar in some instances to those of Sezary cells, were also observed.

II. EM Morphology

The most striking features of the malignant cells in this condition were the irregularities

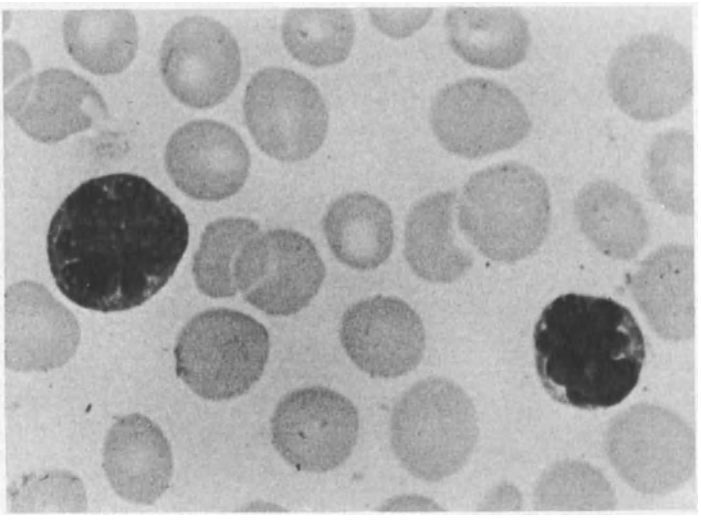

Fig. 1. LM morphology of atypical lymphoid cells

of nuclear outline (Fig. 2). These ranged from notchings or indentations of the nucleus to complex convoluted or lobulated profiles, in some instances resembling the 'cerebriform' nuclei of Sezary cells. Nuclear chromatin was peripherally condensed in the majority of cells, although blast-like cells with little heterochromatin and large nucleoli were present in small numbers in most cases and were predominant in one patient. Active Golgi zones, presence of electron-dense granules (often clustered) and bundles of fibrils (in two patients) were notable cytoplastic features.

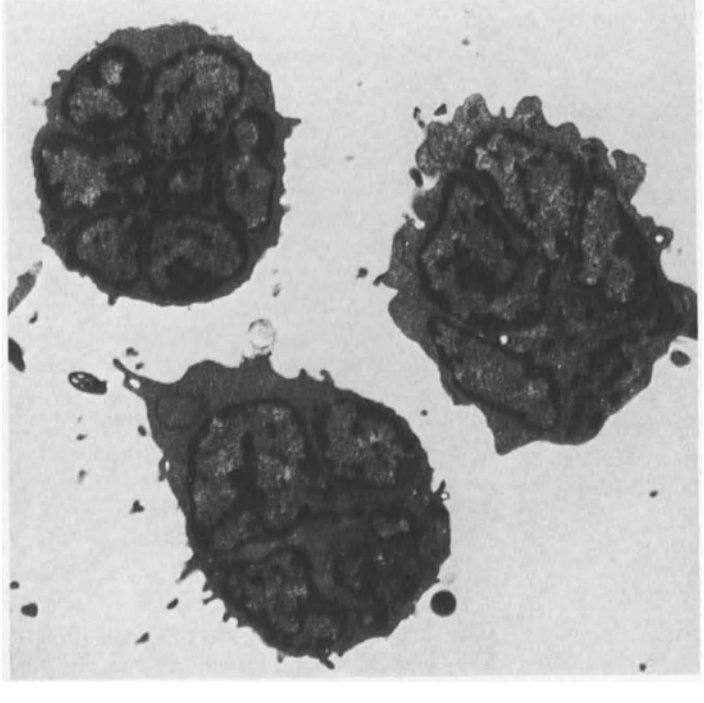

Fig. 2. EM morphology of atypical lymphoid cells. ×5500

III. Cytochemistry

The majority of atypical lymphoid cells displayed positive acid hydrolase reactivity. Although acid phosphatase reactions were positive (tartrate sensitive) in the majority of cells (>70%) in all cases, the intensity and nature of the reaction product was variable. This ranged from weak diffuse positivity in one case to strong multigranular reactivity in another (Fig. 3). Positive ANAE reactions were observed in most (>80%) malignant cells. The reaction pattern was dot-like (single or several discrete granules of reaction product) in the vast majority of cells; occasional cells with weak scattered granular positivity were also noted. β-Glucuronidase reactions were similar to those of acid phosphatase, most cells displaying moderately strong granular positivity. The strongest cytochemical reactions were observed for β-glucosaminidase. Virtually all (>95%) ATLL cells displayed strong multigranular positivity (Fig. 4). Cells from three cases were stained with PAS; in two cases all atypical lymphoid cells were negative; in the remaining case granules or blocks of intensely stained material were observed in >80% of cells.

Table 1. Cytochemical features of ATLL cells

Acid phosphatase	ANAE	β-glucuronidase	β-glucosaminidase	PAS
±/+	±/+	±/+	+	−/+

Reaction intensity: − negative; ± weak; + strong

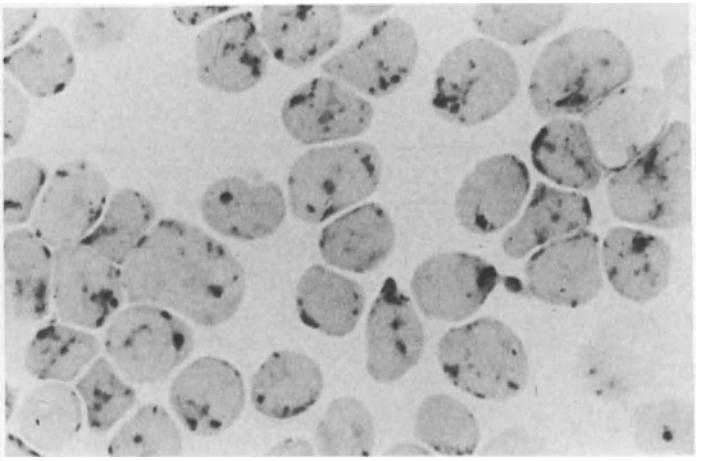

Fig. 3. Acid phosphatase reaction in ATLL cells (cytocentrifuge preparation)

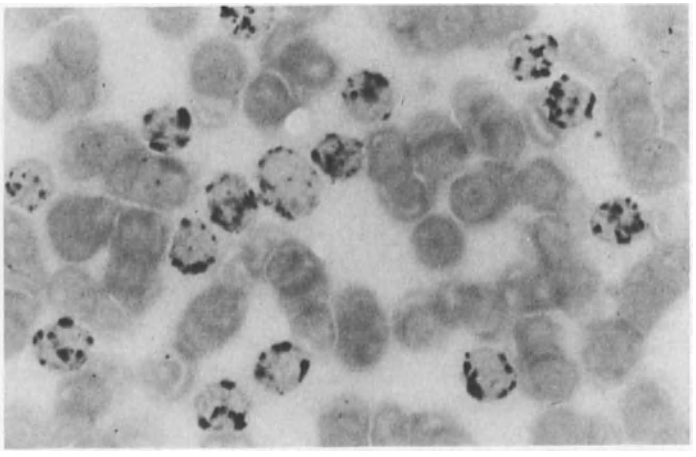

Fig. 4. β-glucosaminidase reaction in ATLL cells

A cytochemical reaction profile of the atypical lymphoid cells in ATLL is shown in Table 1.

D. Discussion

ATLL first described in Japanese patients [4, 5] has more recently been identified in several different racial groups [2, 6–8]. The LM and EM morphological features of the atypical lymphoid cells from PB, BM and lymph nodes in our six West Indian/Caribbean patients bear a close resemblance to those of the Japanese cases [9, 10]: pleomorphic cells with marked nuclear irregularities and condensed heterochromatin, and clustered electron-dense granules. The cytochemical profile of the malignant cells in our patients is consistent with that of a T-lymphoproliferative disorder [3]. The acid phosphatase and ANAE reactions are similar to those described in the Japanese cases [11], with the exception that the acid phosphatase reaction was tartrate sensitive in all our cases but tartrate resistant in the series of Usui et al. [11].

The close similarity in clinical, immunological, morphological and cytochemical features of ATLL as described in different racial groups (Table 2) is intriguing and may be significant in view of the association of HTLV with this disease [12, 13].

Table 2. Similarities between adult T-cell lymphoma-leukaemia in Japanese and West Indian blacks

Morphology Membrane phenotype	} of the neoplastic T cells
Lymph node histology	
High incidence of hypercalcaemia	
Poor prognosis (< 1 year)	
Geographical clustering (Southern Japan, Caribbean basin)	

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Activities and Specificities of *N*-Acetylneuraminyltransferases in Leukemic Cells*

W. Augener, G. Brittinger, W. E. C. M. Schiphorst, and D. H. van den Eijnden

Recently, interest in glycosyltransferases has increased due to the finding that differentiation and neoplastic transformation of cells may be accompanied by alterations in the composition and metabolism of cellular glycoconjugates. Previous studies using lactose as exogenous acceptor have shown apparent differences in the expression of the activities of fucosyl- and *N*-acetylneuraminyltransferases in various leukemic cells arrested at different stages of cell maturation [1].

The aim of this study was to determine the activities and linkage specifities of different *N*-acetylneuraminyltransferases in various leukemic and normal cell populations. Normal and leukemic cells were isolated from human blood by differential and/or gradient centrifugation and characterized by surface membrane marker analysis as described recently [1].

N-acetylneuraminyl(NeuAc)-transferase activites were assayed in cell lysates by measuring the transfer of [^{14}C]NeuAc from CMP-[^{14}C]NeuAc to different exogenous glycoprotein acceptors. The reaction mixture for the standard *N*-acetylneuraminyltransferase assay contained 50 µl lysate (10^8 sonicated cells or platelets/ml of 0.05 *M* HEPES-buffer, pH 6.8, containing 0.1% Triton X-100), 100 µl acceptor glycoprotein solution (2 mg asialo-α_1-acid glycoprotein/acceptor sequence: Gal-β1,4-Glc NAc-oligosaccharide glycoprotein, 1 mg asialo-ovine submaxillary mucin/acceptor sequence: GalNAc-Ser/Thr or 0.7 mg asialo-afuco-porcine submaxillary mucin/acceptor sequence: Gal-β1,3-GalNAc-Ser/Thr in 100 µl of 0.05 *M* HEPES-buffer, pH 6.8), and 2148 pmol/25 µl CMP-[^{14}C]NeuAc (CFB 165 : 50 µCi/ml, spec. activity 291 mCi/mmol, Amersham Buchler) in a total reaction volume of 220 µl. The reaction mixture was made 10 m*M* with $MgCl_2$ and the final concentration of Triton X-100 was 0.023%. After incubation from 0 to 3 h at 37 °C in a shaking water bath, aliquots of 25 µl of the reaction mixture were immediately subjected to high-voltage paper electrophoresis for 70 min at 2000 V in 0.05 *M* sodium tetraborate buffer, pH 9.0, according to Roseman [2], to separate the neuraminylated exogenous glycoprotein acceptors. The paper was cut and the radioactivity due to [^{14}C] NeuAc was determined by scintillation counting. *N*-acetylneuraminyltransferase activities toward exogenous glycoprotein acceptors were expressed as transfer of pmol NeuAc/(h × mg lysate protein). *N*-acetylneuraminyltransferase activity toward endogenous acceptors of the lysates was found to be negligible.

The linkage specificity of the CMP-NeuAc: β-galactosyl [1, 4] *N*-acetylglucosaminide *N*-acetylneuraminyltransferase was establishd after transfer of NeuAc from CMP-NeuAc to the terminal [^{3}H] galactose residues of asialo-α_1-acid [^{3}H] Gal glycoprotein using a micromethodology based on methylation and hydrolysis of the neuraminylated product, followed by analysis of the emerging trimethyl-[^{3}H] galactosides, as described recently [3].

* Dedicated to Prof. Dr. K. D. Bock on the occasion of his 60th birthday

Cell type	Activity (pmol/h × mg lysate protein)	Linkage specificity
Normal T-lymphocytes (>94% E-R positive)		
252	52	α (2,6)
257	94	α (2,6)
473	110	α (2,6)
Normal B-lymphocytes (>91% EAC-R positive)		
253	78	α (2,6)
258	68	α (2,6)
478	110	α (2,6)
Normal platelets		
467	1425	α (2,6) and α (2,3)
526	2373	α (2,6) and α (2,3)
Normal leukocytes		
466	<30	Not detectable
Cell lines		
D.G.-75	840	α (2,6)
MOLT-4	198	α (2,6)
K-562	62	α (2,6)
Leukemic cells		
281 (AML)	59	α (2,6)
400 (AML)	37	α (2,6)
408 (non-T/non-B ALL)	<30	Not done
433 (non-T/non-B ALL)	<30	Not done
249 (T-ALL)	280	α (2,6)
183 (CLL)	709	α (2,6)
274 (IC)	194	α (2,6)
379 (CLL)	320	α (2,6)
431 (CLL)	268	α (2,6)
432 (CC)	115	α (2,6)

Table 1. Activity and specificity of β-galactosyl(1,4)N-acetylglucosaminide-N-acetylneuraminyltransferase in human normal T- and B-lymphocytes, platelets, and various leukemic cells (exogenous acceptor: asialo α_1-acid glycoprotein)

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia (B-cell type); IC, leukemic immunocytic non-Hodgkin lymphoma (LP immunocytoma); CC, leukemic centrocytic non-Hodgkin lymphoma

A. Serum Type of Glycoprotein *N*-Acetylneuraminyltransferase

As shown in Table 1 *N*-acetylneuraminyltransferase activity toward asialo-α_1-acid glycoprotein was low in both isolated normal T- and B-lymphocytes, whereas the transferase activity of various leukemic and culture cell line cells varied from almost not detectable to high.

Leukemic myeloid (No. 281 and 400) and non-T/non-B lymphoid cells (No. 408 and 433) as well as K-562 cells were apparently arrested at an early stage of differentiation and displayed low levels of activity, whereas T-lymphoblasts (No. 249) and the MOLT-4 cells (Thy-ALL) were further developed along the T-cell lineage and transferred 280 and 198 pmol NeuAc/(h × mg lysate protein), respectively. Neoplastic

B-lymphocytes and the D.G.-75 cells (B-lymphoblasts) were further developed along the B-cell lineage, showing varying levels of activity of *N*-acetylneuraminyltransferase of the serum glycoprotein type.

When analyzed for the linkage specificity of this *N*-acetylneuraminyltransferase it appeared that the leukemic and culture cell line cells as well as normal T- and B-lymphocytes exclusively transferred NeuAc to position C-6 of the terminal [^{3}H] galactose residues of asialo-α_1-acid[^{3}H]Gal glycoprotein, indicating that these cells only expressed α2,6-*N*-acetylneuraminyltransferase activity.

However, normal platelets displayed in addition to high α2,6- also high α2,3-*N*-acetylneuraminyltransferase activity.

B. Mucin Type of Glycoprotein *N*-Acetylneuraminyltransferase

When tested with asialo-ovine submaxillary mucin (GalNAc-Ser/Thr) and asialo-afuco-porcine submaxillary mucin (Gal-β1,3-GalNAc-Ser/Thr) as exogenous glycoprotein acceptors, neither the various leukemic cells nor normal T- and B-lymphocytes revealed significant activities [<30 pmol NeuAc/(h × mg lysate protein)] of *N*-acetylneuraminyltransferases of the mucin glycoprotein type.

C. Discussion

Transfer of NeuAc to the labeled terminal [^{3}H] galactosyl units of the acceptor molecule asialo-α_1 acid [^{3}H] Gal glycoprotein can involve the positions C-2, C-3, C-4, and C-6, depending on the specificity of the CMP-NeuAc: β-galactosyl (1,4)*N*-acetylglucosaminide (α2,2-, α2,3-, α2,4- or α2,6-) *N*-acetylneuraminyltransferase involved. Our data establish α2,6-*N*-acetylneuraminyltransferase as the only *N*-acetylneuraminyltransferase of that type in various leukemic cells and normal lymphocytes.

Leukemic non-T/non-B lymphoid cells were almost devoid of this enzyme activity whereas leukemic T and B cells apparently further developed along the T- or B-cell lineages varied from low activity, as found in normal T- and B-lymphocytes, to high activity, indicating that the expression of α2,6-*N*-acetylneuraminyltransferase activity occurs concomitantly with the differentiation of lymphoid cells. However, no evidence was found for an additional α2,3-*N*-acetylneuraminyltransferase which had been detected in fetal calf liver and in human placenta [4], giving rise to speculations on the possible existence in leukemic cells of an oncofetal α2,3-*N*-acetylneuraminyltransferase. Only platelets displayed α2,3-*N*-acetylneuraminyltransferase activity in addition to high α2,6-*N*-acetylneuraminyltransferase activity. These data establish CMP-NeuAc: β-galactosyl (1,4) *N*-acetylglucosaminide α2,3-*N*-acetylneuraminyltransferase as a marker enzyme for human platelets.

Using lectins such as soybean agglutinin (specificity: GalNAc), peanut agglutinin (specificity: Gal-β1,3-GalNAc), and lobster agglutinin (specificity: NeuAc), changes in the carbohydrate moieties of surface glycoconjugates concomitant with differentiation of lymphocytes have been demonstrated, reflecting alterations in the expression of cellular glycosyltransferases. During the process of cell maturation the surface lectin-binding sites exposing terminal GalNAc or Gal-β1,3-GalNAc units are masked by NeuAc residues. However, our experiments did not reveal any activities of *N*-acetylneuraminyltransferases of the mucin glycoprotein type (acceptor specificity: GalNAc or Gal-β1,3-GalNAc) in lysates of normal lymphocytes and of various leukemic cells arrested at different stages of maturation.

Since the expression of α2,6-*N*-acetylneuraminyltransferase activity of the serum glycoprotein type occurs concomitantly with the differentiation of lymphocytes, masking of the described lectin-binding sites on the surface of lymphocyte seems not to be due to direct chemical linkage of NeuAc to either terminal GalNAc or Gal-β1,3-GalNAc units, but rather to a charge or steric effect of NeuAc residues linked to Gal-β1,4-GlcNAc units by α2,6-*N*-acetylneuraminyltransferase of the serum glycoprotein type.

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WT1: A Monoclonal Antibody Reactive with T-ALL but not with Other Leukemias

W. J. M. Tax, M. F. Greaves, H. M. Willems, H. F. M. Leeuwenberg, P. J. A. Capel, and R. A. P. Koene

A. Introduction

There is ample evidence that the different leukemias represent clonal derivatives of cells "frozen" in a specific state of differentiation or maturation [1]. The hybridoma technology introduced by Köhler and Milstein [2] has permitted the production of monoclonal antibodies directed against differentiation antigens. Such antibodies can contribute considerably to our insight into hemopoietic differentiation and malignancy.

Acute lymphoblastic leukemias (ALL) can be subdivided into four subgroups according to membrane markers [3]. In a majority of cases, leukemic cells carry is the common ALL antigen (CALLA), which can be demonstrated with conventional antiserum or with the monoclonal antibody J-5 [4]. The cells from T-ALL patients are reactive with conventional antithymocyte antisera. The rare variant of B-ALL is characterized by the presence of surface immunoglobulin. When the leukemic cells have lymphoid morphology but lack these three markers, the classification "null ALL" has been suggested [3]. The T-ALL subgroup is heterogeneous. Most, but not all, cases are positive for E-rosetting, and sometimes both CALLA and thymocyte antigens are expressed [5, 6]. It would be useful if a monoclonal antibody were available which reacts with all T cells and is T-lineage specific. The antibodies from the OKT series are either not T-cell specific (OKT9, OKT10) or not reactive with the immature T phenotype which is found in the majority of T-ALL cases [7]. Even OKT11A, reactive with the E-rosette receptor [8], is not ideal since it fails to label those T-ALL which are E-rosette negative.

We have produced a monoclonal antibody, termed WT1, which is specific for human thymocytes and T-lymphocytes [9]. This antibody reacts with all thymocytes including the large, terminal deoxynucleotidyl transferase (TdT) positive blasts (Tax, Janossy et al., manuscript in preparation). The reactivity of WT1 with this putative prothymocyte population raised the possibility that this antibody might be useful for the diagnosis of T-ALL, especially the immature (E-rosette negative) cases. WT1 was therefore tested on a panel of human cell lines of different phenotypes, and on a broad panel of leukemic cells. The results obtained indicate that WT1 is specific for cells of the T lineage and is useful for the diagnosis of T-ALL.

B. Materials and Methods

The characteristics of all cell lines used in this study (except Jurkat) have been summarized by Minowada [10]. Jurkat is an E-rosette positive leukemic T-cell line [11]. Cells were cultured with 5% CO_2 in RPMI-1640 medium (Dutch modification, containing both HEPES and sodium bicarbonate) supplemented with 7.5%–15% heat-inactivated fetal calf serum, 2 m*M* glutamine, 1 m*M* sodium pyruvate, and gentamicin (50 μg/ml). Blood and marrow samples from leukemic patients were sent to I.C.R.F., London, from hospitals throughout the United Kingdom. Preliminary data on the production and specificity

of monoclonal antibody WT1 have been reported previously [9]. Hybridoma cells were injected into pristane-treated Balb/C mice and antibody was purified from the ascites by ammonium sulfate precipitation and protein A-Sepharose chromatography. Reactivity of cells with antibody WT1 was evaluated by indirect immunofluorescence using $F(ab')_2$ goat antimouse IgG antibodies labeled with fluorescein. E rosettes were formed by incubating lymphoid cells for 1 h at 4 °C with aminoethylisothiouronium-treated sheep erythrocytes [12].

C. Results and Discussion

When monoclonal antibody WT1 is tested on human cell lines, only cells with T phenotype are reactive (Table 1). Importantly, the antibody also binds to cells with an immature T phenotype like HSB-2 and CEM, which do not form E rosettes.

WT1 appears to be T-lineage specific when tested on leukemic cells (Table 2). All T-ALL, including several cases which are E-rosette negative, react with WT1, but the other types of leukemia do not react. However, in some cases of myeloid leukemias and one case of erythroleukemia, a weak staining was observed. The optimal diagnostic approach for T-ALL would probably be to combine the membrane staining by WT1 with staining of the nuclei by antiserum against TdT [13].

In conclusion, WT1 appears to be a useful monoclonal antibody for diagnosis and monitoring of T-ALL. Furthermore, the antibody is cytotoxic (IgG2a), and incubation of bone marrow cells with WT1 and rabbit complement did not affect the outgrowth of myeloid or erythroid committed progenitor cells (CFU-GM and BFU-E, respectively: 9 and Th. de Witte, personal communication). WT1 might therefore also be useful for the in vitro elimination of T-ALL blasts from bone marrow. This would render autologous bone marrow transplantation feasible as treatment for T-ALL.

Table 1. Reactivity of monoclonal antibody WT1 with human cell lines

Cell line	Origin[a]	Reactivity
CCRF-CEM	T-ALL	+
CCRF-HSB-2	T-ALL	+
JURKAT	T-ALL	+
CCRF-SB	Normal B[b]	–
RPMI 1788	Normal B[b]	–
DAUDI	BL	–
REH	CALL	–
NALM-1	CML-BC (Ph^1)	–

[a] BL, Burkitt's lymphoma; CML-BC (Ph^1), chronic myeloid leukemia in blast crisis, positive for Philadelphia chromosome

[b] Transformed in vitro with Epstein-Barr virus

Table 2. Reactivity of monoclonal antibody WT1 with leukemic cells

Leukemia/diagnosis	WT1 binding	
	+	–
Acute lymphoblastic leukemia		
Common ALL	0	43
Null ALL	0	5
B-ALL	0	1
T-ALL	8	0
Possible T-ALL[a]	7	0
Mature lymphoid leukemias		
T-Sezary	0	1
T-LCL[b]	0	2
B-CLL, PLL, lymphoma	0	20
Myeloid leukemias		
CML	0	2
CMML	0	1
AML	2[d]	17
CML-BC (M)[c]	1[d]	8
E-L	1[d]	3

[a] Possible T-ALL (all diagnosed as ALL): Four cases: DR^-, TdT^+, E^-, Ig^-, $cALL^-$, other T antigens$^-$; One case: DR^-, TdT^+, E^-, Ig^-, $cALL^-$, other T antigens$^+$; One case: DR^-, TdT^-, E^-, IG^-, $cALL^-$, other T antigens$^-$; One case: DR^+, TdT^+, $cALL^-$, $E/T11^+$, other T antigens$^-$,

[b] T-lymphosarcoma cell leukemia ("helper" phenotype)

[c] TdT^- "myeloid" blast crisis

[d] Weak staining

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Dysfunctional Glucocorticoid Receptors in Acute Leukemia *

R. Bell, A. Lillquist, S. Cotter, S. Sallan, and R. McCaffrey

A. Introduction

The obligatory role of specific cytoplasmic receptors as mediators of steroid hormone action is well established. In lymphoid cell culture systems the development or glucocorticoid resistance is almost always due to a mutation to a receptor negative state [1]. Therefore, if the assumption is that glucocorticoid-induced remission in leukemia is by a direct effect on leukemic blast cells, one would predict that the presence of absence of blast cell glucocorticoid receptors would be a major determinant or clinical steroid sensitivity. However, receptor quantization has not correlated with steroid responsiveness [2–5], and in particular, high receptor numbers are not uniformly associated with responsive disease [6].

We have speculated that this lack of concordance between receptor status and clinical outcome may be due to the presence of glucocorticoid binding macromolecules which are physiologically nonfunctional in those leukemias which are glucocorticoid resistant. To test this hypothesis, we have studied certain biochemical and biophysical characteristics of glucocorticoid binders in normal and leukemia cells, to determine if such dysfunctional binding macromolecules exist, and to establish their relationship to therapeutic outcome.

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B. Materials and Methods

I. Cells and Tissues

1. Normal Tissues

Thymus, spleen, and lymph node from 3–5 day old calves were obtained at a local slaughter house. Bone marrow mononuclear cells were harvested from 12-week-old BALB/C mice. Human thymus was obtained from a 7-year-old child undergoing cardiac surgery. Human peripheral blood lymphocytes were harvested on LSM from CPD anticoagulated whole blood from normal adults [8].

2. Leukemia Cells – Humans

Seventy-eight patients with acute leukemia were studied, 73 at first presentation prior to any therapy, and five at first relapse prior to attempting reinduction therapy. No patient had received steroid medication within 2 weeks of study. Thirty-five had acute lymphoblastic leukemia (ALL) (31 new cases, 4 relapses), 25 had acute myelogenous leukemia (AML) (24 new cases, 1 relapse), and 18 had the blast crisis of chronic myelogenous leukemia (CML). The patients ranged in age from 3 years to 72 years. Samples with less than 75% blast cells were not studied. Blast cells were harvested from EDTA-anticoagulated venous blood (61 patients), bone marrow aspirate (11 patients), or both (six patients).

3. Leukemia Cells – Animals

Cells and tissues from 38 domestic cats and 21 dogs with lymphoblastic leukemia –

lymphoma were studied at the Angell Memorial Hospital, Boston, MA. All animals were newly diagnosed and none had received therapy prior to study. Blast cells were harvested from EDTA-anticoagulated LSM-sedimented venous blood or marrow (18 animals) or from histologically involved nodes (41 animals). Twenty-four of these animals were treated with single agent prednisone 2 mg/kg p.o. × 14 days and their response evaluated by standard clinical criteria.

II. Analytical Procedures

The cytosol preparation and labeling procedures used were modified from the broken cell labeling system of Sakaue and Thompson [7], as previously described [8]. The analytical procedures have been described in detail [8].

C. Results

1. Normal Tissues

^{3}H-Triamcinolone acetonide labeled glucocorticoid receptors in normal lymphoid tissues can be resolved into two components by DEAE chromatography: Peak I elutes at 0.04 *M* salt and peak II at 0.22 *M* salt. Figure 1 A shows the elution profile of labeled receptors from human peripheral blood lymphocytes. By glycerol gradient centrifugation peak I is 3.5S and peak II 8.5S. Peak I binds to DNA while peak II has negligible binding. After heat activation peak II alters its coefficient of sedimentation to 3.5S, changes its elution position to 0.04 *M* salt (peak I area) on DEAE chromatography, and acquires affinity for DNA (Fig. 2 A). The DEAE elution profile of labeled receptor from peripheral blood lymphocytes which had been heated at 20 °C for 30 min is shown in Fig. 1 B.

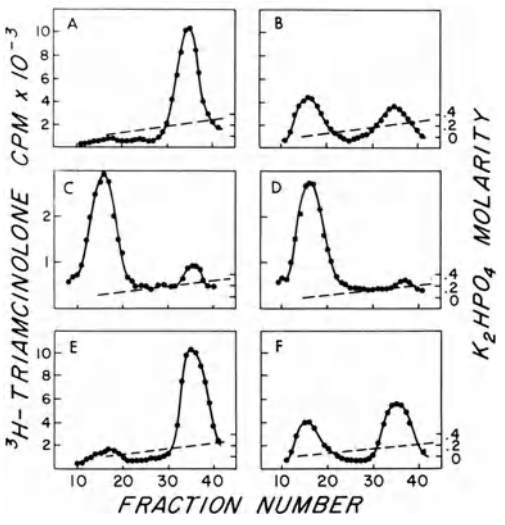

Fig. 1 A–F. DEAE chromatography of ^{3}H-TA labeled glucocorticoid receptors from unheated and heated cytosols. **A** represents the chromatogram obtained from human peripheral blood mononuclear cells; **B** the chromatogram obtained after heating this cytosol (20 °C for 20 min); **C** the chromatogram derived from a case of childhood AML with abnormal single peak pattern; **D** the chromatogram obtained after heating of this cytosol before DEAE chromatography; **E** the chromatogram obtained from a case of childhood ALL; **F** the chromatogram obtained after heating of this cytosol

2. Human Leukemia

In 48 of 78 cases of human leukemias, blast cell ^{3}H-TA binding macromolecules had characteristics identical to those found in

Table 1. Glucocorticoid binders in human acute leukemia

DEAE chromatography	ALL	AML	CML	Total
Normal – two peak	29	15	4	48
Abnormal – single peak	6	10	14	30
Total	35	25	18	78

Table 2. Glucocorticoid binders in animal lymphoblastic disease

DEAE pattern	Responders	Non-responders	Total
Normal Peak I – peak II	9	9	18
Abnormal Single peak	0	6	6
Total	9	15	24

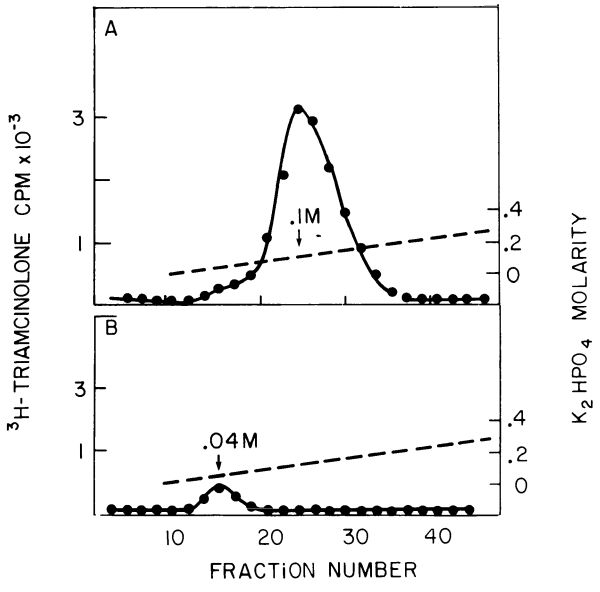

Fig. 2A, B. DNA cellulose chromatography of peak I material from heated cytosols. **A** The elution from DNA cellulose of heat-activated peak I complexes from a case of childhood ALL with the normal tissue DEAE pattern. **B** The elution from DNA cellulose of heated peak I material from a case of childhood AML with the abnormal single peak pattern

normal tissues (Fig. 1 E, F). The receptors from the remaining samples were abnormal (Table 1). The abnormality (30 cases) was the presence of only a single DEAE species eluting in the peak I area (Fig. 1 C, D), which was 2.5S and failed to bind to DNA with or without activation (Fig. 2 B). In two experiments, leukemic cells with the normal DEAE pattern were mixed in a one-to-one ratio with cells showing the abnormal single peak pattern. The binders maintained their identities in this experiment: the "mixed" chromatogram showed peak I and peak II in an additive ratio, suggesting that the single peak pattern is not a product of the labeling or analytic procedures (data not shown).

3. Animal Lymphoblastic Disease

Abnormal ^{3}H-triamcinolone binding macromolecules of the sort seen in human leukemia were identified in the blast cells of 13 of 59 (22%) cats and dogs with this disease; the remaining 46 animals had normal receptors by our criteria. To data, in a prospective blind study 24 aminals (18 with normal receptor patterns, 6 with abnormal glucocorticoid binders) have been treated with prednisone, 2 mg/kg per day $\times 14$ days p.o. None of the six animals with abnormal binders responded, whereas 9 of the 18 with normal receptor characteristics did (Table 2).

D. Discussion

The 78 human leukemic samples we studied by these techniques segregated into two groups: those with normal tissue binder characteristics (48 cases) and those with abnormal binders (30 cases). Normal and abnormal patterns were noted in all three acute leukemia variants (Table 1). Similarly, abnormal binders were found in the blast cells of 13 of 59 domestic animals with lymphoblastic leukemia-lymphoma. In the abnormal cases, the ^{3}H-TA labeled binders eluted as a single species from DEAE in the peak I (0.04 *M* salt) region. The abnormal single peak material did not bind to DNA cellulose, whereas the affinity of peak I material for DNA cellulose was normal in cases in which the initial DEAE profiles were normal. In four of four cases studied, the abnormal single peak DEAE patterns had peak I-peak II S values identical to those of normal tissues (3.5S and 8.5S, respectively). Thus, while the abnormal single peak material cannot be distinguished from normal peak I complexes by DEAE chromatography alone, further characterization on DNA cellulose and glycerol gradients clearly differentiates between the two forms.

Our speculation is that leukemias with abnormal binder characteristics may be incapable of responding to glucocorticoid

therapy. The 78 patients in our series all received multiple agent chemotherapy, and we have therefore made no attempt to correlate clinical outcome with receptor characteristics. In the animal study, none of six animals with abnormal binder characteristics responded. Thus, the abnormalities we have identified to date may provide a biochemical basis for one form of glucocorticoid resistance. Other defects in receptor physiology are now being sought. The establishment of a clearly defined responder phenotype for glucocorticoid receptors in leukemia cells would permit restriction of the use of glucocorticoids to those patients in whom such receptors could be identified, thus eliminating unnecessary exposure to what may sometimes be serious systemic glucocorticoid toxicity. The ability to unequivocally identify physiologically functional receptors would also allow a rational pursuit of a detailed molecular investigation of the mechanisms involved in steroid-induced remissions in leukemia.

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Glucocorticoid-Induced Lysis of Various Subsets of Acute Lymphoblastic Leukemia

N. Galili and U. Galili

A. Introduction

Glucocorticoids are regularly employed in the therapeutic regimes of hematologic malignancies. In attempting to understand partially the therapeutic effect of these hormones, the in vitro cortisol-induced lysis of leukemic cells was studied. In previous studies [2], we have shown that the viability of malignant cells from chronic and acute myeloid leukemias was not affected by 20 h incubation with 10^{-5} *M* cortisol. Chronic lymphatic leukemic cells, however, were readily lysed by cortisol. The cells of acute lymphoblastic leukemia (ALL) patients were divided into two groups; those that were resistant to lysis and those that were sensitive. The purpose of the present study was to correlate sensitivity to cortisol-induced lysis to the phenotype of the ALL cells as defined by monoclonal antibodies and rosetting capacity.

B. Materials and Methods

Thirty-nine patients with ALL were studied. These included new cases at presentation and relapse patients. Most cells were isolated from bone marrow samples, but in several cases in which there were greater than 80% blast cells in the peripheral blood, blood lymphocytes were analyzed.

I. Cell Sensitivity to Glucocorticoids

Briefly, aliquots of 0.2 ml cells (10^6cells/ml) were incubated in flat bottom microwells (Cooke) for 20 h at 37 °C in a humidified CO_2-air (5% : 95%) atmosphere with 10^{-5} *M* cortisol (Ikapharm, Israel). Since a variable proportion of the cells incubated with the glucocorticoids was lysed within 20 h, the amount of cells lysed was assessed by determining the concentration of the remaining viable cells (Trypan-blue exclusion test) in a hemocytometer with a magnification of 400. Their percentage (% lysis) was calculated according to the formula: $(a-b)/a \times 100$, where "a" is the concentration of viable cells in the wells containing medium without steroids and "b" equals the concentration of viable cells in wells containing the drug.

II. Phenotyping of the ALL Cells

The ALL cells were phenotyped using a variety of monoclonal antibodies (see list below) in an indirect immunofluorescent assay and E-rosettes. If the cells typed "common" ALL, they were then examined for the presence of intracytoplasmic IgM (cold acetone-fixed cytospin preparations stained with rabbit antihuman IgM-FITC) to determine whether the cells were of the pre-B phenotype. All cells were also tested with an affinity purified antiterminal deoxynucleotidyl transferase.

Monoclonal antibodies:
J-5 (anti-common ALL [4])
DA-2 (anti-HLA-DR [1])
S33 (anti T cell; produced by Peter Beverly, ICRF, London)
WT-1 (anti T cell [5]).

Cells reacting with either or both S33 and WT-1 are referred to as T^+ cells. Cells that react with DA-2 are noted Ia^+ and those reacting with J-5 are noted J-5^+. Greater than 20% staining is considered positive for the particular antigen.

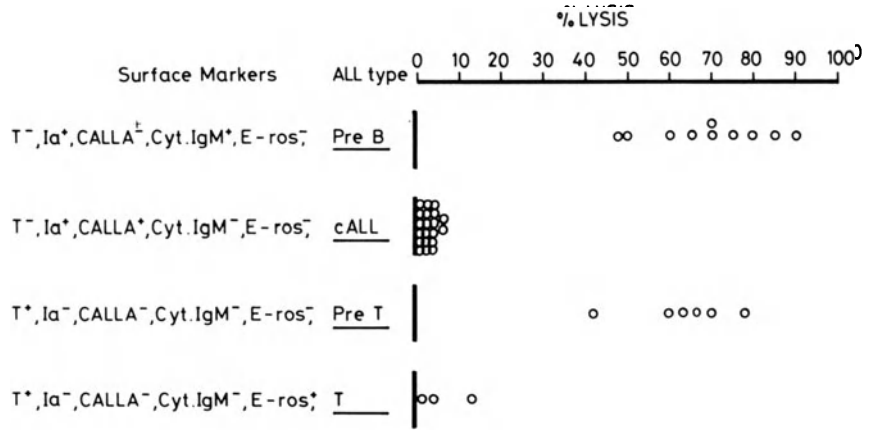

Fig. 1. Cortisol-induced lysis of leukemic cells of various ALL types

C. Results and Discussion

The leukemic cells from 22 patients with common ALL ($J\text{-}5^+$, Ia^+, T^-, E^-) were found to be resistant to lysis following incubation with 10^{-5} *M* cortisol. In contrast, the cells from ten patients with pre-B leukemia ($J\text{-}5^+$ Ia^+ $cIgM^+$ T^- E^-) were readily lysed by cortisol (Fig. 1). Cells from five patients with early or pre-T leukemia ($J\text{-}5^-$ Ia^- T^+ E^-) were similarly sensitive to cortisol-induced lysis, whereas cells from three patients with a more mature T-cell phenotype ($J\text{-}5^-$ Ia^- T^+ E^+) were found to be resistant.

Normal cell populations have been shown to differ in their steroid sensitivity [2]. The human prothymocyte subpopulation was easily lysed by cortisol whereas the thymocytes and peripheral blood lymphocytes were resistant. Interestingly, immunologically activated T-lymphocytes were also found to be sensitive to steroid-induced lysis [3]. Normal bone marrow and polymorphonuclear cells were completely resistant. The steroid sensitivity of the T-ALL cells parallels that of their normal counterparts according to their differentation state. The sensitivity of the pre-B ALL versus the resistance of the cALL cells may likewise be attributed to a differentiation state related phenomena.

The in vivo relavence of this in vitro assay is currently under study. It is suggested that use of this assay may allow for a more efficient use of the steroids in the treatment of leukemic malignancies.

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Epidemiology of HTLV-Associated Leukemia

W. A. Blattner, D. W. Blayney, E. S. Jaffe, M. Robert-Guroff, V. S. Kalyanaraman, and R. C. Gallo

A. Introduction

Type-C retroviruses have long been implicated in the etiology of leukemia and lymphoma in various animal species. Animal models exist for exogenous, horizontal, transmission of these naturally occurring RNA tumor viruses in animals and are especially well characterized for cat, cow, and some other species [21]. Human T-cell leukemia-lymphoma virus (HTLV) is the first type-C retrovirus consistently isolated and associated with specific human malignancies. It is distinct from previously identified animal retroviruses by molecular [15] and immunologic studies [10, 16, 17]. It is an exogenous virus that must be acquired by infection (i.e., not transmitted in the germ line), since HTLV proviral sequences are present in DNA of neoplastic T cells, but not in DNA of nonneoplastic B cells from the same patient [7] or in normal tissues [15].

In this report we summarize clinical and epidemiologic features which suggest that HTLV is etiologically linked to certain malignancies. Our data demonstrate that HTLV is associated with cases of mature T-cell leukemia-lymphoma with common clinical features. These cases tend to cluster in a restricted distribution where HTLV infection is prevalent. Occasional patients from virus nonendemic areas probably acquired HTLV infection prior to developing their T-cell leukemia-lymphoma through travel into HTLV endemic areas and close contact with residents in these areas.

B. Materials and Methods

Samples from various patient and normal populations were submitted as frozen or lyophilized serum or plasma. The clinical and pathologic diagnoses were as recorded by the submitting investigator except for certain cases where pathologic material was reviewed by one of us (E.J.). Serum antibodies to a disrupted whole virus preparation were detected by a modification to the technique previously reported [14]. Natural antibody to the major core protein p24 of HTLV was detected by a radioimmune precipitation (RIP) as previously described [9].

C. Clinical and Pathologic Features

The majority of cases from which HTLV has been isolated, or in which HTLV antibodies have been detected, share many common features. Summarized in Table 1 are the clinical and pathologic characteristics of HTLV-positive cases from the Western Hemisphere and elsewhere (details of HTLV-positive cases from Japan are not included in this report). In all cases in the series, and in cases from Japan [11, 18], HTLV has an association with lymphoma or leukemia of mature differentiated T cells. In current immunopathologic nomenclature, the broad category of malignancies of mature T cells includes T-cell chronic lymphocytic leukemia, cutaneous T-cell lymphomas (CTCL) (mycosis fungoides/Sezary syndrome), peripheral T-cell lymphoma, lymphosarcoma cell leukemia (T-LCL), and adult T-cell leukemia-lym-

phoma (ATL). As shown in Table 1, HTLV is most commonly associated with peripheral T-cell lymphomas (classification in the working formulation [1] as large cell, diffuse mixed, or immunoblastic cell type), TLCL, and ATL. Although two of these cases were classified as CTCL, this association is rare since they are the only two out of over 200 cases from the United States, England, and western Europe that were positive for HTLV serum antibodies.

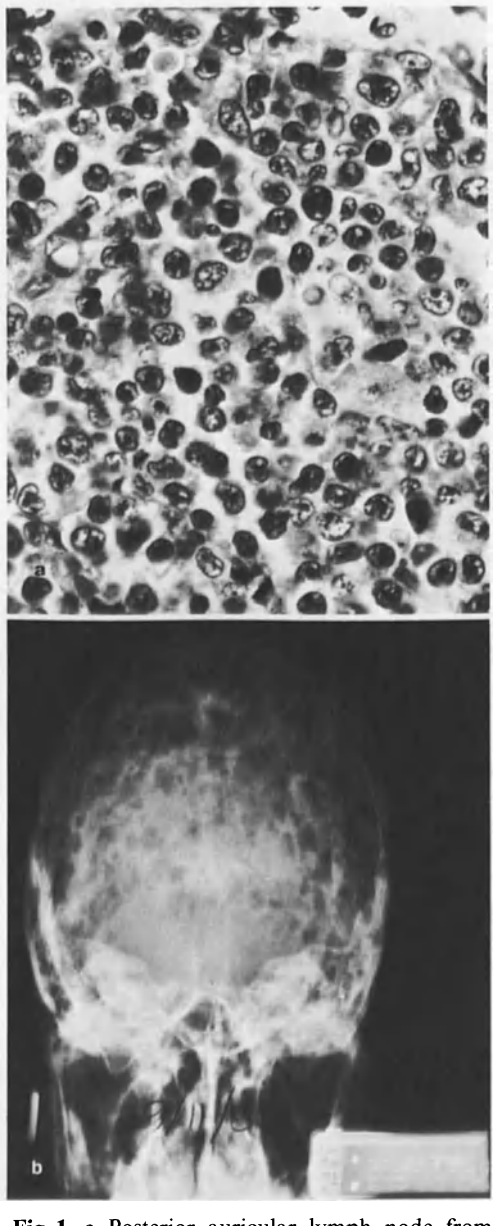

Fig. 1. a Posterior auricular lymph node from case 16. An admixture of small and large atypical lymphoid cells is present. Hematoxylin and eosin, ×430. **b** Plain skull radiograph for case 16. Multiple small lytic lesions are present throughout the calvarium

As is evident from Table 1, the histopathologic diagnoses given vary considerably. There are, however, certain morphologic features shared by most of these tumors. The tissues show diffuse proliferation of a pleomorphic population of lymphoid cells (Fig. 1a). The cells tend to vary considerably both in size and shape. In most cases, cells from all points in the spectrum are present in equal proportions, and such cases are designated as diffuse, mixed cell type by both the Rappaport classification and the working formulation [1]. In other cases, one large lymphoid cell type predominates, and such cases are designated as diffuse large cell, or diffuse large cell, immunoblastic lymphoma, based on the characteristics of the proliferating cells. An inflammatory background is normally not evident.

The histopathologic features described above are similar to those seen with certain other peripheral T-cell lymphomas not associated with HTLV. Thus, there are no specific pathologic features that can be recognized at this point as indicating an HTLV-associated leukemia/lymphoma.

Similarly, there is quite a spectrum in the clinical presentation of cases, which may present as lymphoma, leukemia, or lymphoma with leukemic involvement. Some cases appear associated with fulminant and rapidly progressive disease, whereas others have a much more indolent and chronic clinical course. Thus, the exact relationship of HTLV to a specific clinical entity is far from established. Therefore, systematic surveys in HTLV-endemic and nonendemic areas, with special attention to precise immunologic classification of malignant cells, will be needed to clarify these relationships further. In addition the recent observation of antibody-negative, antigen-positive cases of CTCL (C. Saxinger, personal communication) point to a need for caution in defining virus-disease relationships. Ultimately "molecular" epidemiologic studies of defined disease categories will need to be undertaken before final conclusions can be drawn.

Table 1. Features of HTLV-associated T-cell malignancies

Case No.	Patient	Age/sex	Race[a]	Place of birth	Diagnosis	Clinical signs[b]	Hyper-calcemia	WBC × 10^9 3/liter	Evidence for HTLV	
									Virus isolation	Antibody to HTLV
1	C.R.	28/M	B	Alabama	Pleomorphic CTCL[c]	L, D, O	+	7.1	+	+
2	M.J.	50/M	W	Massachusetts	Sezary syndrome[c]	L, D	–	5.2	+	+
3	M.O.	32/M	W	Washington State	T-hairy cell leukemia[c]	S, H	+	2.9	+	+
4	P.L.	29/F	B	Florida	T-malignant lymphoma, diffuse mixed cell type[c]	L, S, H	+	9.3	N.P.	+
5	E.T.	38/M	B	Virginia	T-lymphoblastic leukemia	L, H	+	235	N.P.	+
6	W.A.	24/M	B	Georgia	T-malignant lymphoma, diffuse mixed cell type[c]	L, D	–	5.8	+	+
7	J.N.	76/M	Aleut	Alaska	Malignant lymphoma diffuse large cell type[c]	S, D	–	4.2	N.P.	+
8	M.B.	64/F	B	St. Lucia	T-malignant lymphoma, diffuse mixed cell type[c]	L, S, H, D	–	435	+	N.P.
9	M.I.	32/F	B	Granada	T-malignant lymphoma diffuse mixed cell type[c]	L, S, H, D, O	–	40	+	+
10	C.M.	31/F	B	St. Vincent	T-lymphosarcoma cell leukemia (ATL)	L	+	39	N.P.	+
11	J.T.	21/F	B	Trinidad	T-malignant lymphoma, diffuse large cell type (ATL)	L, S, H, D, A	+	31	N.P.	+
12	M.H.	49/F	B	Jamaica	T-malignant lymphoma diffuse large cell type (ATL)	L, S	+	17	N.P.	+
13	E.L.	41/F	B	Barbados	T-chronic lymphocytic leukemia (ATL)	L, O	–	40	N.P.	+
14	M.W.	54/F	B	West Indies	T-immunoblastic leukemia (ATL)		+	57	N.P.	+

15	S.W.	45/F	B	Guyana	T-malignant lymphoma immunoblastic type (ATL)	L	+	67	N.P.	+
16	J.S.	33/M	W	Ecuador	T-malignant lymphoma diffuse mixed cell type[c]	L, H, O	+	20	N.P.	+
17	U.K.	45/M	W	Israel	T-malignant lymphoma diffuse large cell type[c]	L, S, H, D, O	+	18	+	+

[a] W, Caucasian; B, black; Aleut, Aleutian Indian

[b] L, lymphnode involvement; S, splenomegaly; H, hepatomegaly; D, skin involvement; A, ascities; O, osteolytic bone lesions

[c] Pathology material reviewed by E.J. (ATL) cases reviewed by Daniel Catovsky, Hammersmith Hospital, London, and although called other diagnoses as noted, represent cases with features of adult T-cell leukemia – lymphoma

One of the striking features of these cases [4], as well as ATL cases from Japan [20], is the frequent occurrence of idiopathic hypercalcemia. In some cases (Fig. 1 b) radiographs show multiple lytic bone lesions mimicking multiple myeloma or metastatic solid tumors. In these cases, no lymphoma cells are seen in association with these lesions but rather osteoclast activity and reactive granulation tissue. This pathologic feature may be a reflection of HTLV-activated lymphokine production, although for cases 4, 16, and 17, the lymphokine, osteoclast activating factor (OAF) was not detectable in bioassay [3]. A variety of lymphokines are produced by HTLV-infected cell lines in vitro, suggesting that integration of proviral DNA results in derepression of host genes including those for various lymphokines [8]. Viral-associated cases with hypercalcemia provide a convenient setting to test this model.

D. Disease Associations and Case Clusters

To evaluate the relationship of HTLV infection and disease, sera from over 900 cases of adult and childhood malignancies from diverse geographic areas have been tested for HTLV antibodies [6]. As shown in Table 2, only 55 of 914 were positive. None of the nonlymphoreticular neoplasms were positive, indicating that HTLV is probably trophic for cells of the lymphoreticular compartment. The bulk of the positives were from patients with definite T-cell malignancies. The remaining nine were from patients with lymphoid and/or myeloid leukemias from Japan. Since some of these cases come from the viral endemic area and/or in some cases have been extensively transfused, the etiologic significance of this association remains to be established.

HTLV is most closely associated with ATL diagnosed in Japan, and 29 of the 34 patients in this series were positive [6]. Among cases of T-Cell non-Hodgkin's lymphoma (T-NHL), 6 of 12 from Japan were also positive. These positive T-NHL cases appear to share features with cases from the United States and elsewhere as recorded in Table 1. The eight cases of HTLV-positive ATL from the Caribbean region (cases 8–15 in Table 1 in this series) confirm the propensity for geographic clusters of HTLV-associated cases to share clinical and pathologic features [2, 4].

Studies of normal populations provide a model for examining the role of HTLV as an etiologic agent. The detection of HTLV antibodies in relatives of cases is of interest since it may reflect the infectivity of HTLV. In Table 3 we summarize the data on members in nine families of HTLV-positive malignancies. Exept for one family from Japan in which four of eight members were antibody positive, in all other cases only one first-degree relative was found to be positive. The fact that both spouses and blood relatives were positive leads us to suspect horizontal rather than vertical

Table 2. HTLV-specific antibodies in lymphoid and nonlymphoid malignancy

Disease	Number positive/number tested by country of origin			
	United States or Europe	West Indies	Japan	Total
T-lymphocytic	9/338[a]	8/9	29/34	46/381
Unclassified and B-lymphocytic	0/258	0/3	4/43	4/304
Myeloid	0/108	0/2	5/27	5/137
Nonlymphoreticular	0/86	N.T.	N.T.	0/86

[a] Cases 16 and 17 in Table 1, although born in Ecuador and Israel respectively, are included here since they were detected as part of a survey of T-cell cases at the National Institute of Health, Bethesda, Maryland, United States

Individuals tested	Number positive/number tested by country of origin		
	United States or Europe	West Indies	Japan
Relatives of HTLV cases	1/8	3/16	13/31
Random donors	4/1120[a]	12/337	39/404[b] 9/509[c]

Table 3. HTLV-specific antibodies in normals

[a] Three of the positives were in blacks from a sample of 130 normals from Georgia. The remaining positive was from a black woman from a sample of 158 normal blood bank donors in Washington, DC

[b] ATL-nonendemic area of Japan

[c] ATL-endemic area of Japan

transmission. Since relatives of these cases shared a common environment, prospective follow-up of exposed relatives of newly diagnosed HTLV-positive cases should help clarify the mode of HTLV transmission [19].

Limited surveys of donors from populations in the United States, western Europe, Japan, and the Caribbean (Table 3) demonstrate that HTLV infection in the general population is limited. Among study subjects from the United States and Europe only three had HTLV antibodies. The three positives were from a sample of specimens submitted to the Georgia State Health Department serology reference laboratory. Many of the United States-born cases of HTLV leukemia-lymphoma, as well as the normal people with HTLV antibodies, are blacks from the southeast United States. This geographic and racial clustering suggests that HTLV infection may have a restricted distribution and/or racial predilection. Further analysis of other populations in this region as well as surveys in other areas of the United States should clarify this relationship [3]. In Japan, sera from ATL nonendemic regions were HTLV antibody negative, while a low prevalence of virus antibody positives was seen in the ATL endemic region of Kyushu (Robert-Guroff et al., unpublished observations). Similarly a population-based survey of normals in the Caribbean revealed a low prevalence of HTLV-positive serology [2].

E. HTLV Infection and Adult T-cell Malignancy

The frustrating failures of virologists in the past to demonstrate and isolate a uniquely human type-C RNA tumor virus etiologically linked with leukemia or lymphoma has led to the belief that retroviruses play no role in these or other human malignancies. Human T-cell leukemia-lymphoma virus (HTLV) is the first human virus of this class consistently identified in association with a specific type of human leukemia-lymphoma [5, 12, 13]. The epidemiologic data summarized here demonstrate that HTLV is associated with several malignancies of the T cell, but not solid tumors or other hematopoietic tumors [6]. Although the role of HTLV as an etiologic agent is not established, it is striking that the distribution of cases is limited to a relatively narrow spectrum of mature T-cell leukemia-lymphoma cases with common features.

In further support of the notion that HTLV and T-cell leukemia-lymphoma are related is the limited distribution of HTLV infection in the general population. Although most dramatic in Japan, where HTLV seropositivity appears geographically associated with clusters of ATL [11, 18], a similar pattern is emerging in the Caribbean [2, 4] and more recently in the southeastern United States blacks [3]. In two cases from the United States (case 2,

MJ, case 3, M.O.) both the clinical and epidemiologic features were distinct from the bulk of other cases. Both patients, however, had histories of travel to and close contact with persons in viral endemic areas. These exceptions to the rule provide further evidence for a virus-disease relationship since it is likely that their HTLV infection was acquired in viral endemic regions. Studies of migrants into HTLV endemic regions may prove especially informative in further delineating etiologic relationships.

In summary, we postulate that HTLV, unlike other putative tumor viruses, is not widespread in distribution but rather is limited to certain regions of the world and within limited areas of some countries (e.g., southwestern Japan). In at least three areas, Japan, the Caribbean, and the southeastern United States, HTLV-associated disease is a mature adult T-cell leukemia-lymphoma. Identification of cases in some other areas of the world suggests that a similar pattern of virus prevalence and disease occurrence will emerge. Thus, although further studies will be needed to establish a causal relationship, the story thus far is similar to that of the retrovirus association with leukemia-lymphoma in animals in which satisfaction of Koch's postulates has established an etiologic role for C-type retroviruses.

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Current Prospects for Clinical Care of Acute Leukemia

E. S. Henderson

Current therapy for acute leukemia can be chosen from among three proven methods and one most promising but preliminary method of treatment: chemotherapy, radiation therapy, bone marrow transplantation, and the up and coming modality of specific immunotherapy. Chemotherapy clearly remains the standard against which all newer treatments must be measured. Bone marrow transplantation relies upon chemotherapy and radiation for maximum effect but has become the treatment of choice for carefully specified patient populations at specific times during their illness. *Specific* immunotherapy (IT) has emerged from the ashes of earlier, and largely catastrophic, trials of nonspecific immunoactive substances to its present state, in which specific reagents not only have potential of their own, but can broaden the scope of bone marrow transplantation through the removal of specific cell populations from the donor cell pool.

Within these modalities exist numerous options, the most important of which are listed in Table 1. These include oral and i.v. chemotherapies at several levels of intensity and complexity, bone marrow allografts of several levels of risk and effectiveness, several forms of immunologically active agents: drugs, antisera, and monoclonal antibodies; and ancillary measures up to and including total body irradiation.

In parallel, the means of identifying different classes of leukemia have been developed along the lines indicated in Table 2. In the process it has become apparent that in terms of the success of therapy the leukemias range from the very responsive, usually curable common ALL with normal chromosome constitutants and minimal evidence for T- or B-cell differentiation (CALLA+, T–, CIg– und SMIg–, normal or hyper-diploid) on the one hand, to Burkitt-like leukemia (TdT–, SMIg+, L3

Table 1. Options for treatment of acute leukemia

Chemotherapy p.o., i.v.
Vincristine, prednisone
Vincristine, prednisone, daunorubicin (or adriamycin), asparaginase
Methotrexate, asparaginase
Standard dose araC
Standard dose AraC, anthracycline ± thioguanine
High-dose araC, asparaginase
High-dose araC, anthracycline
Mercaptopurine, methotrexate
High-dose cyclophosphamide
Intermediate-dose methotrexate
IT methotrexate ± araC ± hydrocortisone
Bone marrow transplant
Isograft
Allograft, HLA and DR identical
Allograft, haplotype identical
Allograft, unmatched, T depleted
Autograft,leukemia cell depleted
Ancillary treatment
Antibiotic prophylaxis
Heparin prophylaxis
Cranial irradiation 18 – 24 Gy
Immunotherapy
Levamisole
Anti-CALLA
Anti T cell (4)
Anti-TdT

Table 2. Identification of different classes of leukemia

Usually curable:
Common ALL, children, hyperdiploid, Ph 1-, CALLA+, sIg and CTg-, CSF-, L_1, or L_2
Occasionally curable:
AML, children, normal karyotype, no prior treatment, M_1, M_2
AML, adult, normal karyotype, no prior treatment, M_1, M_2
Common ALL, adult, hyperdiploid, Phl-, CALLA + SIg and cIg-, L_1 or L_2
APL
Significant palliation and ultimate relapse:
T-cell ALL
AML, adult, abnl, karyotype, no prior R_x
AMoL
ALL, common CALLA+, Ph 1+
ALL, common (pre-B variant) CALLA+, SmIg–, CyIg+
Very refractory to rx:
Blastic crisis, lymphoid
Blastic crisis myeloid
B-cell ALL, SmIg+, L_3, Ia+
Pre-leukemia progressing to AML
AML, AMML, AEL following prior radiation exposure and/or cytotoxic chemotherapy

morphology, ±14q–), myeloid blast crisis (TdT±, SMIg–, CyIg–, Ph 1+ or ++), and other secondary leukemias on the other, with all other types falling somewhere in between the two extremes. A third component of the problem includes patient factors such as race, sex, age, the availability of stem cell donors, and past medical history. On the general level, it is the responsibility of the leukemia therapist to select the appropriate therapeutic options for each combination of leukemic and patient subtype, so as to maximize the likelihood of response, while keeping the risk of acute, chronic, and delayed toxicities to an acceptable minimum.

The current oncological-hematological literature is replete with examples of combined modality treatments including bone marrow transplantation. This volume includes a number of examples (e.g., the papers by Creutzig et al., Hoelzer et al., Thomas et al., Weinstein et al., and Zintl et al.) and also methods of segregating patients into known prognostically significant categories (e.g., the paper by Chen et al. and the overview by Greaves). Taken together one can draw the following *generalities* concerning treatment (Table 3).

The most sensitive class of disease and the form with the best prospect of cure is common ALL in childhood. These individuals usually achieve remission with the combination of vincristine plus prednisone, and are kept in hematological remission with mercaptopurine plus methotrexate. Meningeal and gonadal involvement are frequent in common ALL; CNS prophylaxis must be given in all such cases, and there is evidence accumulating that the use of intermediate-dose methotrexate infusions will further reduce the risk of CNS leukemia and eradicate gonadal leukemia as well.

Common ALL is curable also in a small segment of adults, but appears overall more resistent to both induction and maintenance treatment. The addition of asparaginase and either daunorubicin or adriamycin are required for best response during remission induction. In both children and adults with uncomplicated common ALL bone marrow transplants should be reserved for patients who are in early relapse or who have been reinduced into a second remission, since with initial drug treatment many patients will be cured.

The strategy for good risk AML, AMML, and APL, i.e., those patients without an-

Table 3. Treatment preferences

Leukemia	Initial presentation		First relapse	
	Remission induction	Treatment in CR	Remission induction	Treatment in CR
Common ALL, child	VCR + P	MP, MTX, V, P, CNS XRT + MTX or MD-MTX	VCR, P, ASN, ANT	Allogeneic BMT or autograft with CALLA treatment
Common ALL, adult	VCR, P, ASN, ANT	XRT + Maint IT-MTX or MD-MTX	VCR, P, ASN, ANT or MTX, ASN, VCR, P	
Common ALL, Pre-B	VCR, P, ASN, ANT	TBI, CPA, BMT Allogeneic		
T-Cell ALL	VCR, P, ASN, ANT, AraC, CPA	TBI,CPA, BMT Allogeneic		
B-cell ALL	VCR, P, ANT, ASN, AraC, CPA	TBI, CPA BMT Allogeneic		
AUL	VCR, P, ANT, ASN, AraC, CPA	TBI, CPA, BMT Allogeneic		
Good-risk AML	AraC, ANT, ± TG ± HIDAC, antibiotics	AraC, ANT ± TG (intensive, 6 months) (CNS prophylaxis in children)		
APL	AraC, ANT, ± TG ± HIDAC, antibiotics, heparin	AraC, ANT, ± TG (intensive, 6 months)		
AMML, EL	AraC, ANT ± TG ± VP16, ± HIDAC	AraC, ANT, ± TG (intensive 6 months)	HIDAC, ANT	CPA, TBI BMT
AMOL	VCR, P; AraC, ANT, ± TG, antibiotics, Leukapheresis PRN	AraC, ANT, TG, intensive IT-MTX	± heparin	
Secondary AML	HIDAC	TBI, CPA, BMT		
CML, Blast crisis, lymphoid	VCR, P; HIDAC	TBI, CPA, BMT, or MTX		
CML, Blast crisis, myeloid	HIDAC	TBI, CPA, BMT		

AraC, cytarabine; HIDAC, high-dose cytarabine; ASN, asparaginase; ANT, anthracyclene (daunorubicin, adriamycin); CPA, cyclophosphamide; MP, 6-mercaptopurine; MTX, methotrexate; IT, intrathecal; MD-MTX, intermediate-dose MTX; TG, 6-thioguanine; BMT, bone marrow transplantation; VCR, vincristine; VP16, etoposide

tecedent bone marrow disease or cytotoxic treatment and without major cytogenetic abnormalities (excepting translocation 15:17) is in many ways analogous to that for common ALL. Patients are induced into remission, in this case with araC + anthracycline (7 + 3 or 10 + 3) or ara–C + anthracycline + thioguanine (TAD), and given limited but intensive maintenance therapy. No additional therapy will be required by as many as 30% of responders. For those who relapse a second remission may be achieved by the same drugs, following which allogeneic bone marrow transplantation is performed if a suitable donor is available and if the patient is 40 years of age or less.

For patients with other varieties of acute leukemia, who can be induced into remission but rarely remain there, e.g., T-cell ALL, pre-B-cell ALL, Ph 1+ ALL (or AML), and patients with very high blast cell counts, transplantation should be performed where possible early in the first remission using the most appropriate available resources and techniques.

Until recently, there was little to offer individuals who developed acute leukemia in marrows previously injured by other malignancy, radiation, or cytotoxic drugs. Patients with lymphoid blastic crisis of CML would occasional remit with vincristine ± prednisone, but for the majority, with myeloid secondary acute leukemias, treatment was fruitless. The use of high-dose cytarabine introduced by Rudnick et al. [7] and further evaluated by several groups [1–3] has been recently shown by Preisler and co-workers and Capizzi [1, 6] to be remarkably effective in controlling (albeit transiently) these formerly refractory secondary leukemias (see Table 4). Although remissions to date have been brief, they may permit these individuals to be successful recipients of bone marrow from normal donors, or of autologous marrow from which malignant cells have been eradicated (Ritz et al., Rodt et al., Kersey et al., McCaffrey et al., this volume).

The harnessing of the new techniques of immunodiagnostics and monoclonal antibody production have led to remarkable clinical results in leukemia and lymphoma ([4], Ritz et al., Levy et al., Kersey et al., this volume). Although of recent vintage the effects of antibodies or antisera in vivo, and in vitro in conjunction with autografting, have been so successful that larger trials to confirm and extend these approaches are mandatory. The pursuit of alternative methods of eliminating tumor cells on the one hand or T-effector T cells on the other in order to broaden the application of autografting and allografting for the management of malignancy ([5], Santos, Redt, this volume) are also of high priority.

The ultimate goal for the clinician is to have available a treatment for every type of leukemia in every clinical setting, and a rapid, reliable assay to determine which treatments are required. Progress is being made in this direction ([6], Izzaguira, McCaffrey, this volume). For the present choices must in the main rely on generalities drawn from clinical experience, but progress in assaying and classifying leukemia has been so rapid as to encourage great optimism for the management of acute leukemia in the immediate future.

Table 4. Treatment of secondary leukemias[a] with high-dose cytarabine (HIDIC)

Treatment	No. of patients	No. of CR	No. of PR	Duration (months) of response	Reference
HIDAC	10	6	2	1–4+	[6]
HIDAC + asparaginase	14	9	2	–	[1]

[a] Includes acute leukemia developing in patients diagnosed and treated for CML, polycythemia vera, and preleukemic (myelodysplastic) syndromes

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Mechanism of Malignant Transformation

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Viral Oncogenes and Cellular Prototypes*

P. Duesberg, M. Nunn, T. Biehl, W. Phares, and W.-H. Lee

A. Summary

The structural hallmark of retroviral transforming *onc* genes is a specific RNA sequence that is unrelated to the essential retroviral genes but closely related to certain cellular prototypes termed proto-*onc* genes. Two types of *onc* genes have been distinguished. Type I are *onc* genes which utilize elements of specific sequences only to encode a transforming protein. Type II *onc* genes are hybrids which utilize essential viral (typically *gag*) and specific RNA sequences to encode transforming proteins. Comparisons between viral *onc* genes and cellular proto-*onc* genes are reviewed in the light of two competing models for proto-*onc* function: the quantitative model, which holds that viral *onc* genes and cellular proto-*onc* genes are functionally the same and that transformation is the result of enhanced dosage of a cellular proto-*onc* gene; and the qualitative model, which holds that they are different. Structural comparisons between viral *onc* genes and cellular prototypes have demonstrated extensive sequence homologies in the primary structures of the specific sequences. However, qualitative differences exist in the structure and organization of viral *onc* genes and cellular prototypes. These include differences in promoters, minor differences in the primary structure of shared sequences, and absolute differences such as in the presence of sequences which are unique to viral *onc* genes or to corresponding cellular genetic units. For example, type II hybrid *onc* genes of retroviruses share only their specific but not their *gag*-related elements with the cell, and cellular proto-*onc* genes are interrupted by sequences of non-homology relative to viral *onc* genes. In addition, proto-*onc* gene units may include unique cellular coding sequences not shared with viral *onc* genes. There is circumstantial evidence that some proto-*onc* genes are potentially oncogenic after activation (quantitative model) or modification (qualitative model). Activated by an adjacently integrated retroviral promotor, the cellular prototype of the *onc* gene of the avian acute leukemia virus MC29 was proposed to cause lymphoma and activated by ligation with viral promoter sequences two proto-*onc* DNAs, those of Moloney and Harvey sarcoma viruses, were found to transform mouse 3T3 cell lines. Mutations presumably conferred 3T3 cell-transforming ability to the proto-*onc* gene of Harvey sarcoma virus that has been isolated from a human bladder carcinoma cell line. In no case has an unaltered proto-*onc* as yet been shown to be necessary and sufficient for carcinogenesis. Despite this and structural differences between viral *onc* genes and cellular proto-*onc* genes, we cannot at present conclusively distinguish between the quantitative and the qualitative models because a genetic and functional definition of most viral *onc* genes and of all cellular prototypes of viral *onc* genes are not as yet available.

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B. Definition of *onc* Genes

Over *15* transforming *onc* genes have been identified in retroviruses since the discovery of the *src* gene of Rous Sarcoma virus (RSV) in 1970 [3, 8]. The only known function of *onc* genes is neoplastic transformation of normal cells to cancer cells. The structural hallmark of all retroviral *onc* genes is a specific RNA sequence that is unrelated to the three essential virion genes, *gag, pol,* and *env.* Thus, *onc* genes are not essential for retroviruses and instead may be viewed as molecular parasites. Retroviruses with *onc* genes are inevitably and immediately oncogenic in susceptible cells or animals. However, retroviruses with *onc* genes are rare and appear only sporadically in natural cancers [13, 37]. The majority of naturally occurring retroviruses lack *onc* genes and are therefore not directly oncogenic. Retroviruses without *onc* genes carry the three essential virion genes *gag, pol,* and *env* and

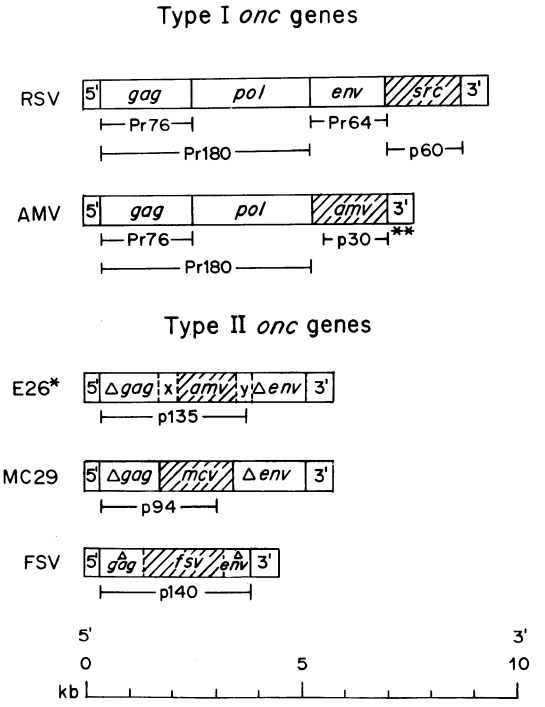

Fig. 1. Genetic structures of oncogenic avian retroviruses with two different types of *onc* genes: Type I *onc* genes utilize specific sequences unrelated to the three essential virion genes *gag, pol,* and *env* [8] to encode transforming proteins. Type II or hybrid *onc* genes utilize specific and virion genes, typically *gag*-related sequences, to encode transforming proteins. *Boxes* indicate the mass of viral RNAs in kilobases (kb) and *segments with in boxes* indicate map locations in kilobases of complete or partial (Δ) complements of *gag* and *env*, of the *onc*-specific sequences (*hatched boxes*) and of the noncoding regulatory sequences at the 5′ and 3′ end of viral RNAs. *Dotted lines* indicate that borders between genetic elements are uncertain. The three-letter code for *onc*-specific RNA sequences extends the one used previously by the authors: *src* represents the *onc*-specific RNA sequences of Rous sarcoma virus (RSV); *fsv* is that of Fujinami sarcoma virus (FSV); *mcv* that of the myelocytomotosis virus (MC29); and *amv* that of the Avian myeloblastosis virus (AMV), which is shared by erythroblastosis virus E26 [3, 4]. Recently, a different nomenclature has been proposed by others, i.e., *myc* (=*mcv*), *myb* (=*amv*), *fps* (=*fsv*) [40]. *Lines and numbers under the boxes* symbolize the complexities in kilodaltons of the precursors (Pr) for viral structural proteins and of the transformation-specific polyproteins (p). For E26 (*) a complete genetic map is not yet available. *X* and *Y* represent unidentified genetic elements of E26 [4]. The protein product of AMV (**) has only been identified in cell-free translation assays (Lee and Duesberg, unpublished), and the size of p30 is deduced from the proviral DNA sequence [29]. The size of the p94 protein of MC29 is deduced from the proviral DNA sequence (Papas et al., this volume) and is at variance with the p110 value reported previously [3]

are found primarily as nonpathogenic parasites which are transmitted horizontally, congenitally, or through the germ line in many animal species. However, certain animals, and, as recently shown, man (Gallo et al., this volume), which carry such viruses turn viremic and develop leukemias and other forms of cancer after long latent periods. Because of their association with leukemias these viruses are often referred to as leukemia viruses [3, 8, 13, 37, 40].

Only one viral *onc* gene, the *src* gene of RSV, is genetically defined by classical deletion and recombination analysis [3, 8]. The *onc* genes of all other retroviruses are associated with defective viruses which lack functional complements of all (or most) essential virion genes. Thus *onc* deletions of defective viruses are not functionally detectable and recombinants cannot readily be distinguished for lack of secondary markers. Consequently all viral *onc* genes except for *src* are not genetically defined.

Nevertheless, on the basis of structural and product analyses, two types of *onc* genes have been distinguished: Type I *onc* genes utilize their specific sequences and viral regulatory sequences to produce unique transforming proteins unrelated to other viral gene products (Fig. 1). Type II *onc* genes are hybrids containing specific sequences and elements of essential virion genes (typically from the *gag* gene, which encodes the core proteins of retroviruses). Together these elements encode hybrid-transforming proteins, which are the basis for the definition of hybrid *onc* genes (Fig. 1) [21]. Examples of type I *onc* genes in the avian tumor virus group are the *src* gene of RSV, which encodes a p60 protein (protein of 60,000 daltons) with an associated kinase function, and the *amv* gene of avian myeloblastosis virus (AMV), which probably encodes a p30 protein (Fig. 1) [29]. Type II *onc* genes are encoded by defective viruses like the acute leukemia viruses MC29 and E26 and like Fujinami sarcoma virus (FSV). The type II *onc* genes of these viruses encode *gag*-related, nonstructural, and probably transforming proteins p94 (MC29), p135(E26), and p140(FSV) (Fig. 1).

To date *onc* genes have not been found in any other group of viruses, such as DNA tumor viruses, which when oncogenic appear to transform with essential virion genes [8]. Genes with exclusive oncogenic function have also not been identified in normal cells. However, genes with oncogenic potential have been isolated from cancer cells (see below).

C. The Qualitative and the Quantitative Model

Retroviruses with *onc* genes represent a paradox among viruses in that they appear only rarely in nature and there is no evidence for horizontal spread. Explanations were offered by the oncogene [15] and protovirus [36] hypotheses which stated that prototypes of *onc* genes exist in some latent form in normal cells and may be induced and transduced by retroviruses without *onc* genes. The original oncogene hypothesis was formulated in 1969, based on sero-epidemiological evidence. Since reverse transcriptase and infectious proviral DNA [37, 40] had not yet been discovered, the hypothesis could not conclusively define the nature of cellular oncogenes and possible mechanisms of transduction by retroviruses. This was first attempted by the protovirus hypothesis [36] and subsequently by a revised oncogene hypothesis [36a].

Using *onc*-specific hybridization probes to test this hypothesis, DNA sequences related to viral *onc* genes have been found in normal animal cells [12, 30, 33]. Some of these sequences, termed proto-*onc* genes, were shown to be highly conserved in different animal species including drosophila [31a, 32, 34]. However, the function of proto-*onc* genes is unknown and proto-*onc* genes, like most viral *onc* genes, have not as yet been genetically defined. Therefore efforts to elucidate the relationship between proto-*onc* genes and viral *onc* genes is, at this time, limited mainly to structural analyses. Analysis of functional relationships has to await genetic definition and functional identification of gene products.

There are two competing views of the role of proto-*onc* genes in normal cells: the *quantitative model,* which postulates that viral *onc* genes and cellular prototypes are the same and the transformation is due to enhaced gene dosage as a consequence of

virus infection [1, 2] and the *qualitative model,* which holds that viral *onc* genes and cellular prototypes are functionally different [3, 8, 10]. The quantitative model sees normal cells as potential cancer cells with switched off *onc* genes. The qualitative model postulates mutational change and possibly deletions of the coding sequence to convert a cellular gene into a viral *onc,* or possibly a non-viral cancer gene. Obviously the two views have very different implications for possible prevention and therapy of tumors caused by such genes, with the qualitative model offering better opportunities for a therapeutic approach. In the following we discuss studies to distinguish between the two models which focus on (a) structural comparisons of molecularly cloned cellular proto-*onc* genes and viral *onc* genes, (b) on measuring expression of proto-*onc* genes in normal and tumor cells, and (c) on testing morphological transforming function of cloned DNAs in transfection assays on cultured mouse 3T3 cell lines.

D. Structural Relationship Between Viral *onc* Genes and Cellular Prototypes

Structural comparisons at the nucleic acid sequence level between type I and type II viral *onc* genes and cellular prototypes of different avian tumor virus subgroups have provided the following insights:

The primary sequence of the type I *src* gene of RSV, and of proto-*src,* are very similar if compared by hybridization and heteroduplex analyses [19, 31, 33]. However, scattered single base changes are detected by mismatched regions in *src* RNA-proto-*src* DNA hybrids [19]. By contrast, the organizations of viral and cellular *src* sequences are quite distinct. Heteroduplex analyses of molecularly cloned viral *src* DNA and cellular proto-*src* DNA show that the cellular sequence is interrupted by six to seven sequences of nonhomology compared with the viral counterpart [25, 31, 35]. If one assumes that (i) the coding sequences of the cellular proto-*src* locus and of viral *src* are the same and (ii) that the regions of nonhomology are noncoding introns and (iii) that the single base changes reflect silent or conservative mutations, proto-*src* could have the same function as *src.* Since there is as yet no direct proof for these assumptions, one cannot clearly distinguish between the two models on a structural basis [3, 19]. Basically, the same limitations regarding a distinction between the two models also apply to structural comparisons of other type I *onc* genes with cellular prototypes.

For example, the *onc* gene of Moloney sarcoma virus, v-*mos*, was shown to contain five and its cellular prototype, c-*mos*, 21 unique 5′ codons in addition to 369 codons shared by the two genes [26 a, 38 a].

Recently, we have compared the type II *onc* gene of MC29, the first hybrid *onc* gene identified in retroviruses [21], with its cellular prototype. A heteroduplex formed between molecularly cloned MC29 DNA and a molecular clone of the cellular prototype of the MC29-specific sequence shows that the specific sequence of 1.6 kb termed *mcv* has a complete counterpart in the cellular locus and that the cellular sequence is not flanked at its 5′ end by a *gag*-related element (Fig. 2) [10, 28]. This has been confirmed by biochemical analyses [28]. The heteroduplex also shows that the proto-*mcv* sequence is interrupted by a 1-kb sequence of nonhomology (Fig. 2). Thus, even if one assumes that the internal sequence of nonhomology is a noncoding intron (see Papas et al., this volume), the cellular proto-*mcv* could not encode the p94 *Δgag-mcv* hybrid protein encoded by MC29 (Fig. 1).

The same appears to be true for the cellular prototype of the hybrid *onc* gene of FSV, which also lacks a *Δgag* element (Fig. 3). The cellular prototype of the FSV-specific sequence (*fsv*) is interrupted by only minor sequences of nonhomology if compared with the 5′ 2 kb of the viral counterpart ([20]; Lee, Phares and Duesberg, unpublished). Since the cellular prototypes of type II *onc* genes are not linked to *gag* or other essential retroviral genes, it follows that type II hybrid *onc* genes are qualitatively different from their cellular prototypes.

Due to the absence of direct genetic and biochemical evidence it may be argued that the *Δgag* element of the hybrid *onc* genes found in MC29, FSV, E26 (Fig. 1), and

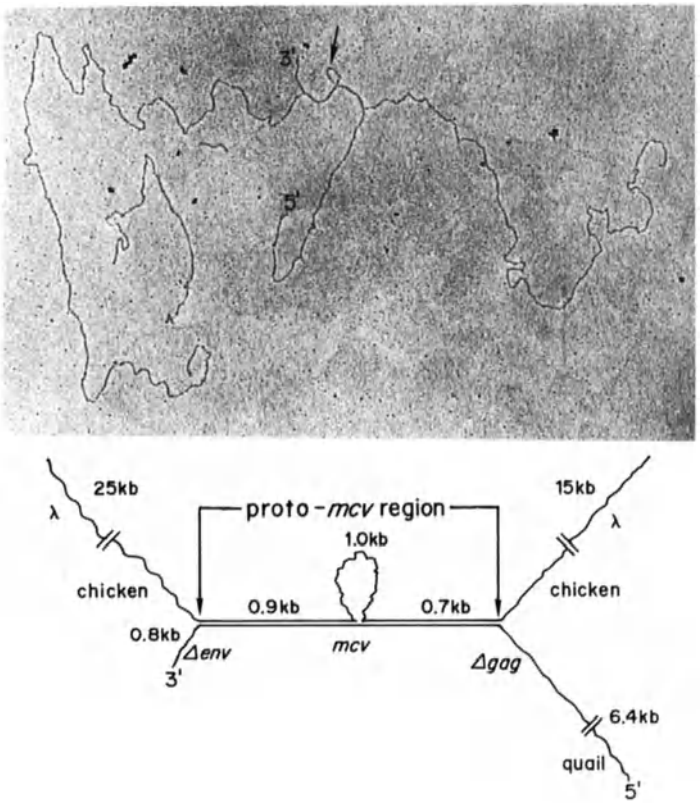

Fig. 2. Electron micrograph of a heteroduplex formed between a fragment of molecularly cloned MC29 proviral DNA and proto-*mcv*, the cellular MC29-related locus of the chicken cloned in lambda phage. Procedures for heteroduplex formation and analysis have been described [28]. The MC29 proviral DNA used was a restriction endonuclease *Eco*RI-resistant DNA fragment that extends from the 5′ end of the viral DNA into *Δenv* (see Fig. 1 for a complete genetic map of MC29). DNA of the proto-*mcv* clone includes the MC29-related locus flanked by about 6–7 kb of chicken DNA at eigher side and then by the two arms of the lambda phage vector. The *arrow* marks the 1-kb sequence of nonhomology that interrupts the MC29-related sequence of proto-*mcv*. The diagram reports length measurements of the respective DNA regions of the heteroduplex in kilobases (data are from Duesberg et al. [10] and Robins et al. [28]

many other avian and murine acute leukemia and sarcoma viruses [3, 40] is not necessary for transforming function. However, several observations lend indirect support to a distinctive role for *Δgag* in hybrid *onc* genes: (a) The genetic *Δgag-x* design is highly conserved in *onc* genes of different taxonomic groups of viruses [3, 40] consistent with a functional role of *Δgag* in hybrid *onc* genes. In support of this view, Temin et al. have recently shown that *gag* may not be essential for packaging of some viral RNAs by helper virus proteins and thus would not necessarily be conserved for this purpose [38 b]. (b) Since *Δgag* together with the specific sequences of a given oncogenic virus forms one genetic unit, i.e., the hybrid *onc* gene which is translated into one nonstructural, probable transforming protein, *Δgag* is also likely to play a direct role in *onc* gene function. If *Δgag* were not necessary for oncogenic function, viruses would have evolved where *Δgag* would not be translated, e.g., spliced out from a mRNA at the posttranscriptional level.

A distinctive role for *Δgag* in *onc* gene function is illustrated by one peculiar pair of *onc* genes which share the same specific sequence but not *Δgag*. One of these, the *onc* gene of AMV, appears to utilize the specific sequence (*amv*) only to encode a

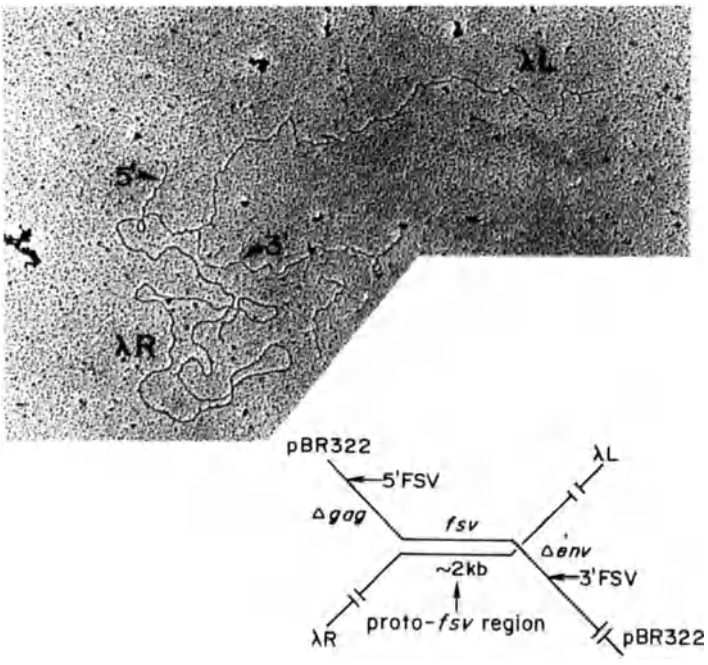

Fig. 3. Electron micrograph of a heteroduplex formed between Fujinami sarcoma virus (FSV) proviral DNA molecularly cloned in the plasmid pBR322 [20] and proto-*fsv*, the chicken cellular locus related to the FSV-specific sequences (*fsv*) (Lee and Duesberg, unpublished). Isolation of the proto-*fsv* sequence from a chicken DNA library in lambda phage followed procedures published previously by this laboratory [28]. Procedures for heteroduplex formation were those described for Fig. 2. The proto-*fsv* lambda phage used here shares about 2 kb with FSV DNA which maps adjacent to *Δgag* in FSV. The 2-kb region of the cellular proto-*fsv* locus appears colinear with its viral counterpart. It is as yet unclear whethter proto-*fsv* represents all FSV-specific sequences, unrelated to essential retrovirus genes, or whether additional proto-*fsv* specific sequences exist that would map between the 2-kb region and *Δenv* of FSV ([20]; Lee, Phares and Duesberg, unpublished)

type I transforming protein although AMV contains a complete *gag* gene (Fig. 1) ([9, 29]; Papas et al., this volume). The other, the *onc* gene of E26, utilizes *Δgag* together with *amv* to encode a type II hybrid-transforming protein (Fig. 1) [4]. The different *onc* gene structures of AMV and E26 correspond to different oncogenic properties. AMV causes exclusively myeloblastosis and E26 causes primarily erythroblastosis [22]. Thus the *onc* genes of AMV and E26 have distinct functions consistent with distinct *onc* gene structures although they share a related specific sequence (*amv*). Extrapolating from this, one can imagine that the proto-*amv* sequence together with adjacent cellular information may be part of a gene with again a distinct cellular function. The same may be true for the functional relationship of all hybrid *onc* genes with their cellular homologs.

Further it appears that related viral *onc* genes and cellular prototypes may differ in the amount of a shared, specific sequence. For example, the specific sequences of the hybrid *onc* genes of MC29 and its relatives MH2 and CMII [3] or of Fujinami and PRCII sarcoma viruses [3, 17, 41] may differ as much as 30% from each other. Likewise the *amv* sequences of AMV and E26 differ in complexity, with E26 lacking both 5′ and 3′ *amv* sequences (Nunn and Duesberg, unpublished). This argues that subsets of a cellular sequence may be sufficient for transforming function as part of a viral transforming gene. By contrast the high degree of conservation of proto-*onc* genes in vertebrates and invertebrates [31a, 32, 34, 40] argues that all cellular sequences, related to a given class of viral hybrid *onc* genes, are necessary for their unknown cellular function including those sequences which

are not shared by all viral *onc* genes of a given class.

Comparison with cellular prototypes indicates that hybrid *onc* genes have at least two essential structural domains one represented by the minimal complement of a given class of specific sequences shared with a cellular locus, the other by *Δgag*. Moreover, the cellular genes may in addition to the codons shared with viral *onc* genes consist of other cell-specific codons that together have a function that is different from viral *onc* genes. These differences suggest, but do not prove, that the products encoded by viral hybrid *onc* genes and the genes of the cellular proto-*onc* loci have different functional domains.

E. Expression and Biological Activity of Proto-onc Genes: Evidence for a Role in Carcinogenesis?

A direct assay of the function of cellular proto-*onc* genes is not yet available. In addition it has not as yet been possible to isolate proto-*onc* genes from normal cells that are directly oncogenic. Consequently, no cancer has as yet been shown to be caused by a proto-*onc* gene.

Nevertheless, there is circumstantial evidence that cellular proto-*onc* genes have oncogenic potential. For example, it has been speculated that proto-*onc* genes may be activated by promotors or enhancers of retroviruses without *onc* genes [14, 26]. Such promoters are encoded in viral LTRs, the terminal sequences of proviral DNA and may function like the promotors of bacterial IS-elements [29a]. Applied to retroviruses, the hypothesis states that such activation requires integration of the provirus adjacent to proto-*onc* and subsequent transcription of a hybrid mRNA which includes at its 5′ end viral LTR sequences and cellular proto-*onc* sequences downstream [14, 38]. Thus, the viral promoter would activate cellular genes located downstream of the provirus. This hypothesis would explain how the rather ubiquitous retroviruses without *onc* genes may occasionally become oncogenic. If correct, this would lend direct support to the quantitative model.

Accordingly, virus-negative tumors [11] and tumors induced by nondefective retroviruses without *onc* genes have been screened for the expression of sequences related to viral *onc* genes [14, 16, 26]. Specifically, enhanced expression of proto-*mcv* (Fig. 2) by promoters of avian leukemia viruses without *onc* genes has been proposed to cause bursal lymphoma in chicken after latent periods of over 6 months [14]. However, this proposal raised a number of questions: (a) for example, why does activated proto-*mcv* not cause the acute myelocytomatosis, carcinoma, or sarcoma caused by MC29? This difference may signal qualitative differences between the functions of viral *onc* genes and the hypothetical oncogenic functions of cellular prototypes. These differences may reflect the structural differences, namely linkage of *mcv* to *Δgag* in the viral but not in the cellular gene. It is recognized that this explanation implies that proto-*mcv* has potential oncogenic function, albeit different from the *onc* gene of MC29. However, evidence listed under (c) and (e) suggests that proto-*mcv* may neither be necessary nor sufficient for lymphomagenesis. (b) A recent reinvestigation of proto-*mcv* activation by avian leukemia viruses has revealed that activation also works upstream and as well as in the opposite polarity within a region of about 20 kb flanking proto-*mcv* [26]. Although this does not rule out activation of proto-*mcv* as the cause of the lymphoma, it rules out a common and orthodox mechanism to explain the reportedly causative activation of proto-*mcv*. (c) This work and the original study also raise the questions why proto-*mcv* activation was only observed in 80% of retroviral lymphomas and thus may not be a necessary condition for lymphoma and why the latent period for leukemia virus to cause bursal lymphoma would be at least 6 months [14]. Considering the high multiplicities of infection, the large number of bursal cells, and a complexity of 10^6 kb of the chicken genome, a successful infection within 20 kb of proto-*mcv* should be a rather frequent event consistent with a short, rather than a long, latent period for leukemogenesis. (d) Furthermore, it is unclear why in other cases of viral leukemias, it has not been possible to demonstrate promotion of cellular genes [16] and why a correlation between neoplasia and enhanced expression of

known cellular proto-*onc* genes in a number of virus-negative human tumors cannot be demonstrated [11]. (e) An attempt to isolate directly the presumably activated oncogenic proto-*mcv* gene from bursal lymphoma cells has led to the detection of a transforming DNA that is unrelated to MC29 [5]. In these experiments DNA isolated directly from tumor cells has been tested for oncogenic function on the mouse fibroblast 3T3 cell line. Assuming that the 3T3 cell assay is suitable to detect a leukemogenic transforming gene, as has been suggested in some cases ([27]; Lane et al., this volume), this result means that proto-*mcv* was either not responsible for the bursal lymphoma at all [14] or that upon activation it played an indirect role. In the latter scenario, proto-*mcv* could mutate the cellular gene identified in the 3T3 assay to create a maintenance gene for lymphoblast transformation [5]. If correct, the experiments that detected proto-*mcv* activation in lymphoma [14] would have found a lymphoma initiation gene by searching for the presumed maintenance gene with a probe for the acute *onc* gene of MC29. It would appear that available evidence does not prove that proto-*mcv* activation is necessary or sufficient for lymphomagenesis.

There is circumstantial evidence that some other proto-*onc* genes become oncogenic upon activation. Using the techniques of DNA transfection two proto-*onc* genes, i.e., those related to the murine Moloney and Harvey or Kirsten sarvoma viruses, have been shown to transform mouse 3T3 cells after ligation to viral promoter LTR sequences derived from Moloney or Harvey sarcoma virus [6, 23]. Although this does imply that these proto-*onc* genes are potentially oncogenic, the relevance of this result to nonviral cancer is uncertain (a) because the cellular loci are not normally linked to viral LTRs and are only oncogenic after ligation with sarcoma viral LTRs, (b) because the genes of the proto-*onc* loci and their products are not yet genetically and biochemically defined and thus are not directly comparable to their viral counterparts, and (c) because to date the assay has been restricted to the 3T3 cell line, which is pre-neoplastic and transforms spontaneously or can be transformed by a large number of viral and nonviral DNAs [27, 39]. It is on the basis of this assay that the structural differences between the v-*mos* and c-*mos* [26 a, 38 a] are considered functionally irrevelant [1]. Moreover, to date the same assay has not shown transformation potential for over a dozen other proto-*onc* sequences from normal cells including proto-*src*, which, upon transfection, was expressed at high levels in mouse cells yet failed to transform these cells morphologically (Shalloway and Cooper; Parker and Bishop, personal communication). In particular not a single prototype of a hybrid *onc* gene like proto-*mcv* was shown to have transforming function despite similar efforts (Robins and Vande Woude, personal communication).

Recently, DNA has been isolated directly from cell lines derived from human tumors and has been tested for oncogenic function in the 3T3 cell assay system. In some cases transforming DNA was extracted from bladder carcinoma cells with properties of a proto-*onc* gene. This DNA resembled the *onc* gene of Harvey and Kirsten sarcoma viruses [7, 24]. Since the DNA equivalent of normal cells did not transform 3T3 cells it would follow that a mutational change must have converted this human proto-*onc* gene to become active in the 3T3 cell assay. However, not all cell lines prepared from bladder tumors yielded active DNA, and DNA from primary tumors has not as yet been tested. It remains to be shown that the DNA that was active in the 3T3 cell assay also caused the original cancer.

It would follow that consistent with the qualitative model there is as yet no direct functional or genetic evidence to prove a direct role of proto-*onc* genes in carcinogenesis. Normal proto-*onc* genes have only been shown to be oncogenic on 3T3 cells after modification. In one case proto-*onc* genes were ligated to viral LTRs. In the other case mutation presumably conferred transforming ability to the proto-*onc* gene related to Harvey sarcoma virus isolated from a human bladder carcinoma cell line. Proto-types of type II *onc* genes have not as yet been positive in the 3T3 cell assay and the bursal lymphomas reportedly caused by activation of proto-*mcv* are qualitatively different from the tumors caused by the type II *onc* gene of MC29. Indeed, some re-

cent results suggest that these lymphomas are maintained by a transforming gene that is unrelated to proto-*mcv*. Taken together these may be signals that viral *onc* genes and their cellular prototypes are qualitatively different.

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The Transforming Gene of Avian Acute Leukemia Virus MC29

K. Bister, P. Enrietto, T. Graf, and M. Hayman

A. Introduction

The transforming gene of avian acute leukemia virus MC29 is represented by a hybrid structure of a partial complement from the structural gene *gag* and MC29-specific sequences, termed *myc*. As a genetic unit these sequences together code for the transforming protein p110 [1–3]. From the continuous MC29-transformed quail cell line Q10 [3] partially transformation-defective (*td*) mutants of MC29 have been isolated which have a strongly reduced ability to transform macrophages in vitro or to induce in vivo any of the tumors typically associated with wild-type (*wt*) MC29 [9]. These mutants are still able to transform fibroblasts in culture. It was recently shown that their altered oncogenic properties are due to the loss of transformation-specific sequences from the genomic RNAs and their protein products [5, 8]. On passage through chicken macrophages, one of these mutants, MC29 *td* 10H, gave rise to a virus, termed MC29 10H BI, which has regained the ability to transform macrophages efficiently [11].

In this communication we report on nucleotide-sequencing data which make possible a more precise location of the deletions in the *td* MC29 *myc* region, and we also show that MC29 10H BI RNA contains *myc* sequences that are not present in *td* MC29, but are shared with *wt* MC29 as well as with the cellular *c-myc* locus.

B. Results and Discussion

I. Genetic Structure of *td* Mutants of MC29

The basic genetic structure of the deletion mutants *td* 10A, *td* 10C, and *td* 10H is shown in Fig. 1. The genomic RNAs have lost overlapping *myc*-specific sequences of 200, 400, and 600 nucleotides, respectively. The deleted sequences are represented by *myc*-specific oligonucleotides 1, 7b, and 26, located 3′ to a *Sal*I site present in *wt* MC29 proviral DNA [5]. In order to locate these deletions more precisely we have sequenced *wt* MC29 proviral DNA and were able to locate *myc* oligonucleotides 26 and 1 near to the *Sal*I site and *myc* oligonucleotide 3 near to the *myc-env* junction (Fig. 2). All *td* mutant RNAs contain *myc*-oligonucleotide 3 and *env* oligonucleotides 7a, 14a, and 2 [5]. Hence, the *myc-env* junction is unaffected by the deletions and conserved in *td* and *wt* MC29 RNAs. All *td* mutant RNAs lack *myc* oligonucleotide 26 [5], and their proviruses lack a *Cla*I site present in *wt* MC29 proviral DNA ([6]; compare Fig. 2). The smallest deletion, in mutant *td* 10A, appears to end within the 5′ half of oligonucleotide 1, since 10A RNA was shown to contain a truncated version (1a) of this oligonucleotide, differing from the *wt* form by the lack of one U and one AAC residue upon digestion with RNase A [5]. This would place the 5′ origin of the deletion near the *Sal*I site. In excellent agreement with this the larger deletions in *td* 10C and *td* 10H result in the total loss of oligonucleotides 1 and 7b, which apparently maps 3′ from 1 and was found in *td* 10A RNA [5].

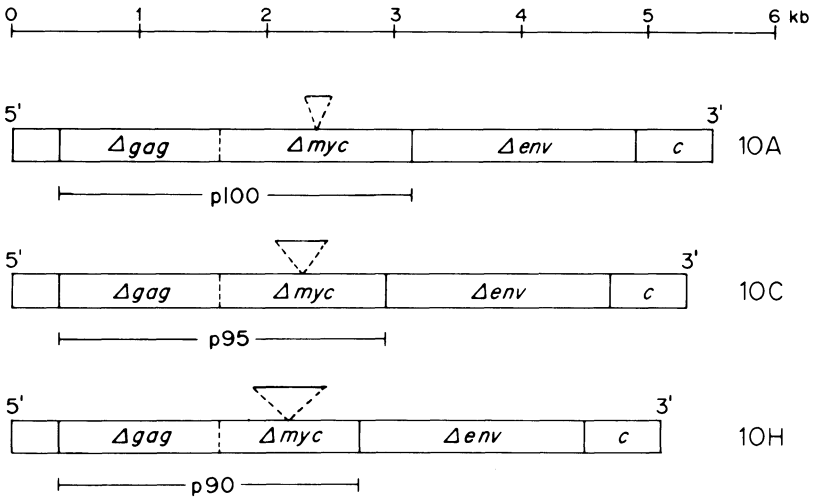

Fig. 1. Schematic diagram of the genomic RNAs and gene products of MC29 deletion mutants 10A, 10C, and 10H. *Δ* indicates the presence of partial complements of the structural genes *gag* and *env*, and of the transformation-specific sequences *myc*. Complexities of RNAs and genes are given in kilobases (kb), those of the *gag-myc* hybrid proteins (p) in kilodaltons

Fig. 2a,b. Nucleotide sequence analysis of *wt* MC29 proviral DNA around the *Sal*I and *Cla*I sites in the center of the *myc* region **a** and around the *myc-env* junction **b**. **a** A subcloned 1.5-kb *Pst*I fragment of cloned MC29 DNA [13] was sequenced from both the *Sal*I and the *Cla*I sites by the method of Maxam and Gilbert [7]. Sequences corresponding to previously identified *myc*-specific T_1-oligonucleotides 26 and 1 (in the 5′ to 3′ order) are underlined [1, 5]. **b** A subcloned 1.1-kb *Sal*I-*Bam*HI fragment of cloned MC29 DNA [5] was sequenced from the *Bam*HI site. Sequences corresponding to previously identified *myc*-specific T_1-oligonucleotide 3 are indicated by a *solid line*; those corresponding to *env* T_1-oligonucleotides 7a, 14a, and 2 (in the 5′ to 3′ order) are indicated by *broken lines*

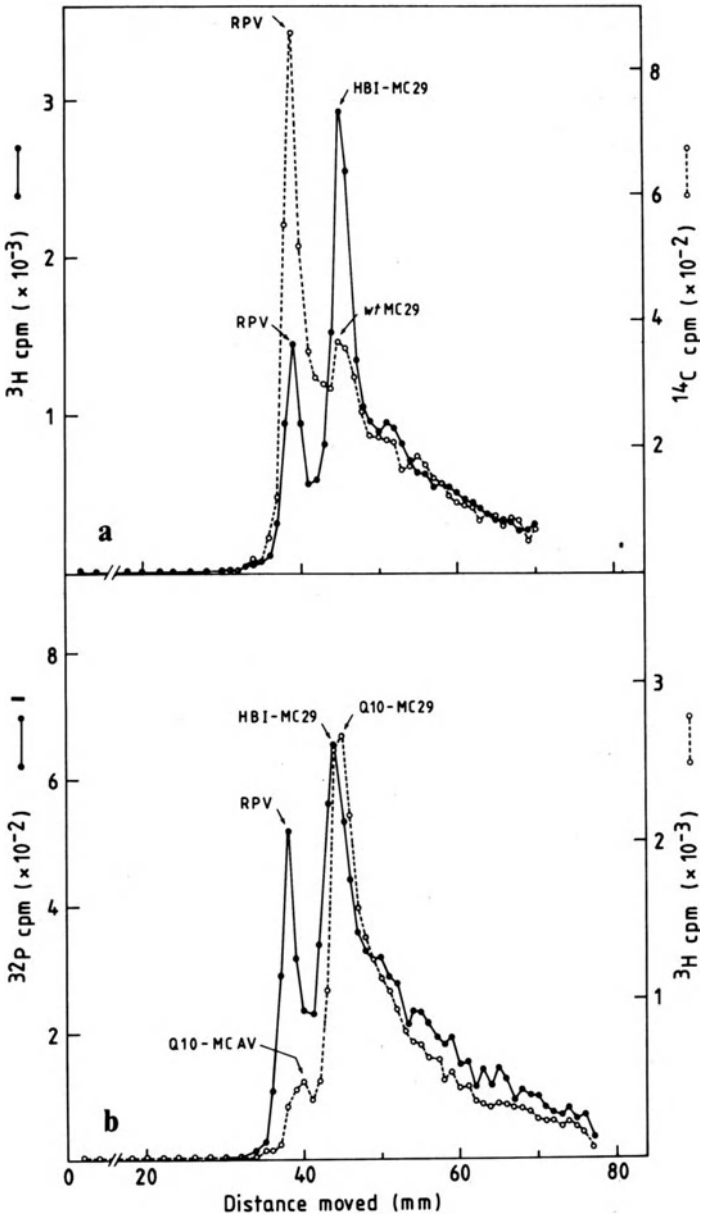

Fig. 3a,b. Electrophoresis in 2.1% polyacrylamide gels of the RNA monomers of **a** [^{3}H] or **b** [^{32}P] 10H BI (RPV) in the presence of **a** [^{14}C]RNA of *wt* MC29 (RPV), rescued from the nonproducer line Q8, or **b** [^{3}H]RNA of Q10-MC29 (Q10-MCAV). Electrophoresis was at 50 V for 4 h and otherwise as described [5]

These data allow the following conclusions: (1) The deletions in all three *td* mutants are overlapping and probably even start from the same map position near the *Sal*I site. (2) Since the *Sal*I site maps about 2050 nucleotides (corresponding to protein sequences of 78,000 daltons) 3′ from the initiation site for the synthesis of the *gag*-related transforming proteins [5], it appears almost certain that the deletions are in phase and that the transforming proteins (p100, p95, p90) of the *td* mutants share the carboxy-terminal sequences with the *wt* protein (p110). This is also strongly supported by the fact that the sizes of the deleted RNA sequences correspond closely to the smaller sizes of these proteins (Fig. 1). (3) Since all mutants were selected for fibroblast transformation, it appears that the 5′ or 3′ terminal *myc* sequences, or both, are needed for that activity, but not the central *myc* sequences. Their deletion,

however, strongly affects the potential for hematopoietic cell transformation. This could possibly reflect the presence of two functional domains on the *wt* p110, one of which would be deleted in the mutant proteins. A possibly important difference between the *wt* and the mutant proteins is that p110 is strongly phosphorylated whereas the mutant proteins have lost specific threonine phosphorylation sites [4, 10].

II. Genetic Structure of Recovered MC29 10H BI

When *td* MC29 10H was passaged through chick macrophage cultures, a virus (10H BI) was recovered that had regained the ability to transform macrophages as efficiently as *wt* MC29 [11]. Here, we used a nonproducer line of 10H BI-transformed quail embryo fibroblasts that was superinfected with ring-necked pheasant virus (RPV) and labeled with [^{3}H]uridine or $H_3{}^{32}PO_4$. Virus was purified and electrophoretic analysis of purified, heat-denatured viral RNA showed the presence of two components: the 8.5-kb RNA of RPV and a 5.7-kb RNA, comigrating with *wt* MC29 RNA originating from MC29-transformed cell lines Q8 or Q10 (Fig. 3). Hence, 10H BI RNA is 0.6 kb larger than the genomic RNA of the deletion mutant *td* MC29 10H (Fig. 1) from which it was derived on passage through macrophages. To analyze the structure and possible origin of these acquired sequences, T_1-oligonucleotide fingerprints of [^{32}P] RNA were prepared. [^{32}P] 10H BI (RPV) RNA was hybridized with cloned proviral MC29 DNA from the *Δgag-myc* or the *myc-Δenv* region [5], or with cloned *c-myc* DNA [12]. Figure 4 shows that the oligonucleotide pattern of 10H BI RNA from the *myc* and the adjacent *gag* and *env* regions is very similar to that of *wt* MC29 RNA reported previously [5]. In particular, *myc* oligonu-

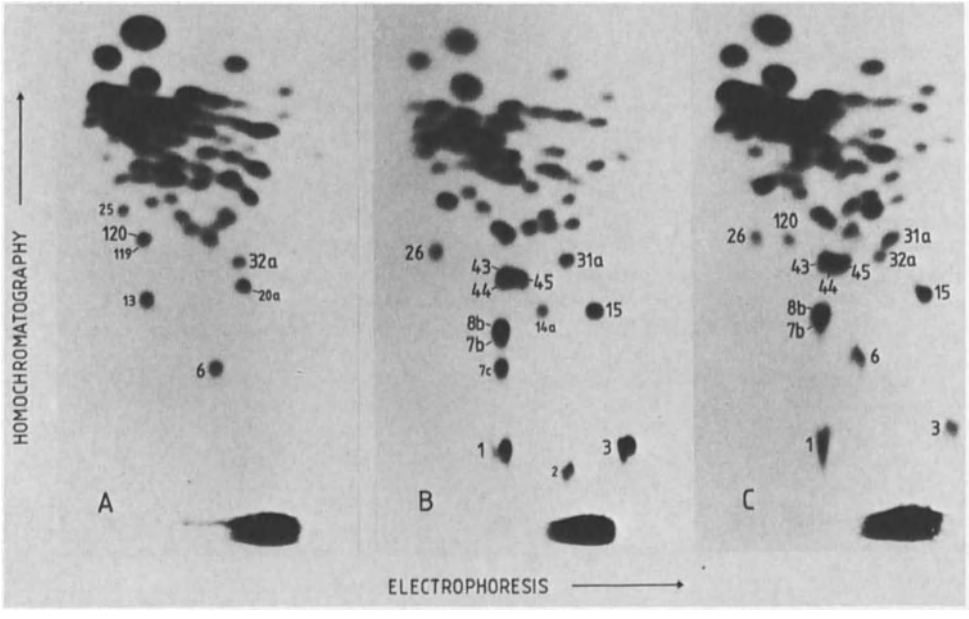

Fig. 4A–C. Fingerprint analysis of [^{32}P]RNA from 10H BI (RPV) hybridized with (**A**) DNA from pBR322 containing the 5′ half of the *myc* sequence and adjacent *gag* sequences (*pmyc*-5′; see [5]), (**B**) DNA from pBR322 containing the 3′ half of the *myc* sequence and adjacent *env* sequences (*pmyc*-3′; see [5]), or (**C**) DNA from phage Charon 4A containing the cellular *c-myc* locus [12]. Hybridization of [^{32}P]RNA with plasmid or phage DNA, isolation of the hybrid, and fingerprint analysis of hybridized RNA were carried out as already described [5]. The composition of T_1-oligonucleotides after digestion with RNase A was determined. Oligonucleotides with previously found compositions were numbered accordingly [5]. The composition of 7c is: 2U, 6C, 2AC, AU, AG, AAC. *Large numbers* indicate *myc* oligonucleotides; *small numbers* refer to *gag* or *env* oligonucleotides

cleotides 1, 7b, and 26, which are missing from *td* MC29 10H RNA are present in 10H BI RNA. Oligonucleotide 7c appears to be a variant of *env* oligonucleotide 7a present in *wt* and *td* 10H MC29 [5]. There are more differences in the oligonucleotide pattern of *td* 10H and 10H BI RNAs (not shown). These would have to be explained by point mutations or recombination with helper virus, if 10H BI is directly derived from *td* 10H in a recombinational event involving cellular *c-myc* sequences. Due to the close relationship between the viral and the cellular *myc* sequences [12, 13], such an event would involve double legitimate recombination, and hence could conceivably have occurred during the generation of 10H BI.

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Cellular *onc* Genes: Their Role as Progenitors of Viral *onc* Genes and Their Expression in Human Cells

F. Wong-Staal, S. Josephs, R. Dalla Favera, E. Westin, E. Gelmann, G. Franchini, and R. C. Gallo

Type C retroviruses are associated with naturally occurring leukemia-lymphomas in many animal species, including man (see Gallo et al., this volume), and they are also the first tangible tools for approaches to our understanding of the molecular mechanisms of cellular transformation (see Duesberg et al. and Vande Woude et al., this volume). While most retroviruses isolated in nature are slow acting in disease induction (chronic leukemia viruses), a subclass of viruses, including the sarcoma viruses and acute leukemia viruses, cause disease rapidly in vivo and transform appropriate target cells efficiently in vitro. These properties are conferred on the viruses by a viral transforming (*v-onc*) gene. There are now at least 17 different *v-onc* genes identified in retroviruses isolated from avian, rodent, feline, and primate species. All viral *onc* genes are derived from normal cellular genes (*c-onc* genes) of their host of origin. *C-onc* genes share several common features: (1) they are highly conserved among all vertebrates and some are conserved even in nonvertebrate species. For example, sequences related to a few *onc* genes have been identified in *Drosophila* [8], and an enzyme related to $pp60^{src}$ of Rous sarcoma virus has been detected in sponge (M. Schartl, personal communication). (2) With few exceptions, the homology between *c-onc* and *v-onc* genes is interrupted by nonhomologous stretches in *c-onc*, tentatively referred to as introns. (3) Most *c-onc* genes have been found to be expressed at least at some stages of normal cell growth, suggesting they are functional genes in normal cellular processes.

The interest of our laboratory in *c-onc* genes is chiefly in their role in growth, differentiation, and neoplastic transformation of human cells. We have molecularly cloned human DNA sequences homologous to a number of viral *onc* genes ([2, 3]; Franchini et al. [5] and unpublished), and in collaboration with others we have studied their patterns of expression [4, 10, 11]. Through the studies on the structures of the human cellular genes and their relationship to the corresponding viral genes we have gained some insight on the possible mechanism of generation of the acutely transforming viruses. In the first part of this paper we will selectively present a longitudinal study comparing the viral and cellular counterparts of one particular *onc* gene, namely *sis*, the *onc* gene of simian sarcoma virus, to illustrate this point. Studies on the expression of *onc* genes in human cells have also shed some light on the cellular role of these genes, and in the second part of this paper we will summarize studies on the expression of six *onc* genes in different human cells.

A. The Transforming Gene of Simian Sarcoma Virus (*sis*)

Simian sarcoma virus, SSV, is the only acutely transforming primate retrovirus characterized to date. It was isolated in association with a helper virus, SSAV, from the fibrosarcoma of a woolly monkey [12]. Closed circular SSV and SSAV viral DNA intermediates were cleaved with a one-cut enzyme and ligated to phage vector arms to

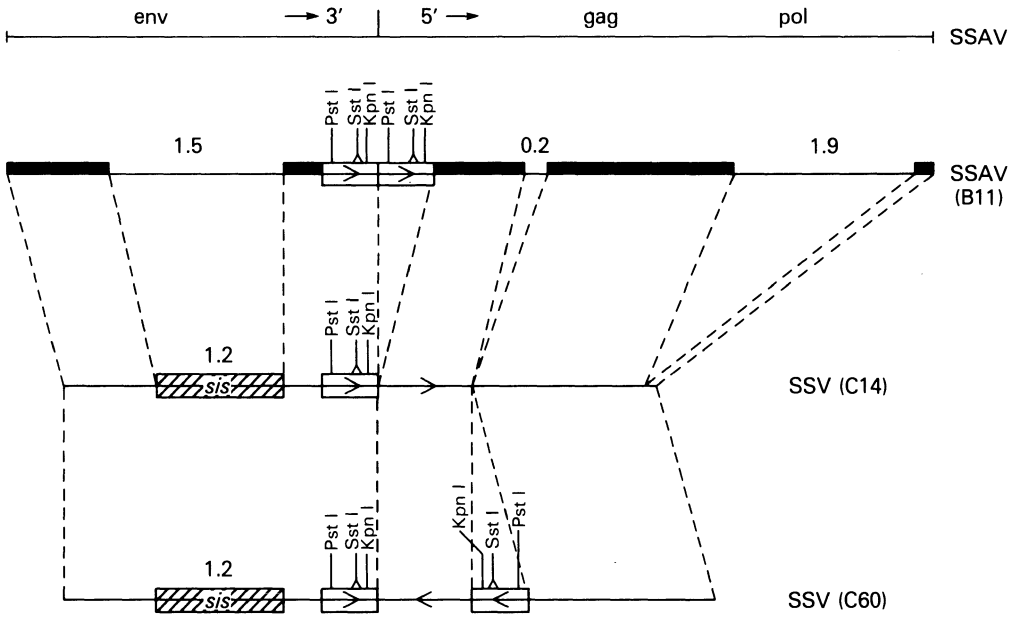

Fig. 1. Genetic structures of SSAV and two molecular clones of SSV

generate clones of complete, permuted SSV, and SSAV genomes, which were compared by restriction enzyme and heteroduplex mapping [6]. The SSAV DNA genome is a 9.0-kb molecule with two long terminal repeat units (LTR). Two SSV clones from viral DNA intermediates were extensively analyzed (Fig. 1). When compared to SSAV the two clones share three regions of deletion and one substitution: a 0.2-kb deletion near the beginning of the *gag* gene, a 1.9-kb deletion probably comprising most of the *pol* gene, and a 1.5-kb deletion in the *env* gene where a substitution of 1.0-kb of SSV-specific (*v-sis*) sequences is found (Fig. 1). Comparison of *sis* to other viral *onc* genes showed no detectable homology [13]. Hybridization of *v-sis* sequences to DNA from different primate and nonprimate species showed that *v-sis* has highest homology to woolly monkey DNA [13]. Since SSV was originally isolated from a pet woolly monkey which cohabited with a pet gibbon ape, and since SSAV is highly homologous to isolates of gibbon ape leukemia virus (GaLV), we concluded that SSV arose from a recombination between a woolly monkey cellular gene and a retrovirus of the GaLV group transmitted from the gibbon to the woolly monkey.

We have determined the nucleotide sequence of the entire *v-sis* region and adjacent SSAV-derived sequences (Josephs et

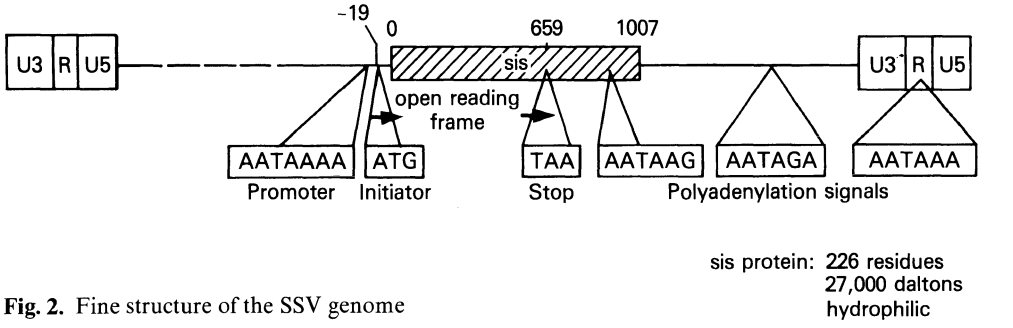

Fig. 2. Fine structure of the SSV genome

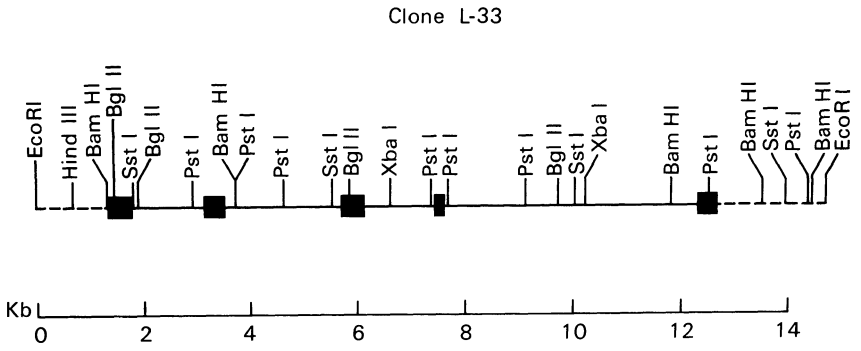

Fig. 3. Organization of human *c-sis* locus. Regions of homology with *v-sis* are represented as *black boxes*

al., submitted). The results revealed a single large open-reading frame which initiates 19 bases within the helper-derived sequences and terminates about two-thirds into *v-sis* (Fig. 2). The translated protein product is highly hydrophilic and has a molecular weight of 27,000 daltons. The first six amino acid residues of the *v-sis* protein are coded by the SSAV-derived sequences. The recombination of *v-sis* and SSAV occurs within a triplet at the 5′ end. Potential promoter sequences AATAAAA are found upstream from the ATG initiator codon and in the U3 region of the LTR. It is not clear which is the functional promoter for the *sis* protein.

B. The Human Cellular Homologue of *sis*

Labeled *v-sis* sequences detects a single locus in human DNA [14], and an RNA transcript of 4.2-kb in some human tumor tissues [4, 10]. A clone of the human *c-sis* gene was isolated from a recombinant phage library [2]. The DNA insert of this clone (L33) contains all the *v-sis* specific sequences. Two techniques were used to locate the regions of homology: restriction endonuclease mapping and heteroduplex formation between L33 and an SSV clone. Both analyses revealed that the 1.0-kb of *v-sis* homologous sequences in L33 span a

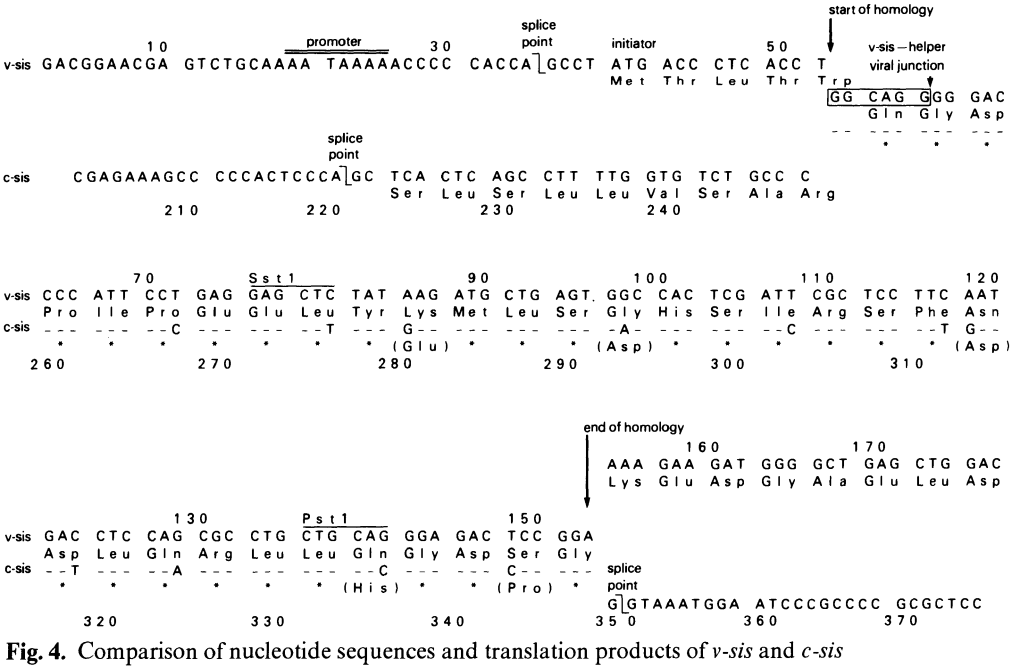

Fig. 4. Comparison of nucleotide sequences and translation products of *v-sis* and *c-sis*

region of 12 kb and are interrupted by at least four nonhomologous regions (Fig. 3). The 5′ region of L33 delimited by the *Eco*RI and *Pst*I sites was subcloned, and a portion of its nucleotide sequence, comprising sequences upstream from and including the entire first homologous region as well as part of the adjacent intron, was determined [3]. When aligned with the *v-sis* sequences, the first *c-sis* homologous region is found to be 1000 bases long also starting within a triplet, and contains six silent base changes and five base changes resulting in altered amino acid residues from *v-sis* (Fig. 4). These changes probably represent divergence of the human and woolly monkey genes rather than that of viral and cellular genes. There is no initiator ATG or promoter sequences within the region of homology or within the 246 bases upstream. However, there is a possible splice site 29 bases upstream of the start of the region of homology. These results taken together suggest that recombination of SSAV and *c-sis* occurred within an internal exon of the functional *c-sis* gene and that *c-sis* consists of additional exon(s) at the 5′ region. These results may also explain why the RNA transcript detected in human cells is much larger than the 1.0-kb *v-sis* gene. An additional interesting finding is the detection of a six-basepair sequence (boxed in Fig. 4) that is found in both *c-sis* and SSAV right at the junction of recombination. This sequence homology may be essential for the recombination event as described in the following model.

C. A Model for Generation of Transforming Retroviruses

Based on the nucleotide sequence data, we present a model for generation of SSV from recombination of SSAV and *c-sis* sequences (Fig. 5). This model may also apply to formation of other transforming retroviruses. First a nondefective (helper) virus infects a cell and integrates at a site on the chromosome. This initial event may be random. If by chance a short sequence in this provirus is homologous to a cell sequence downstream, recombination between these sequences may delete out the

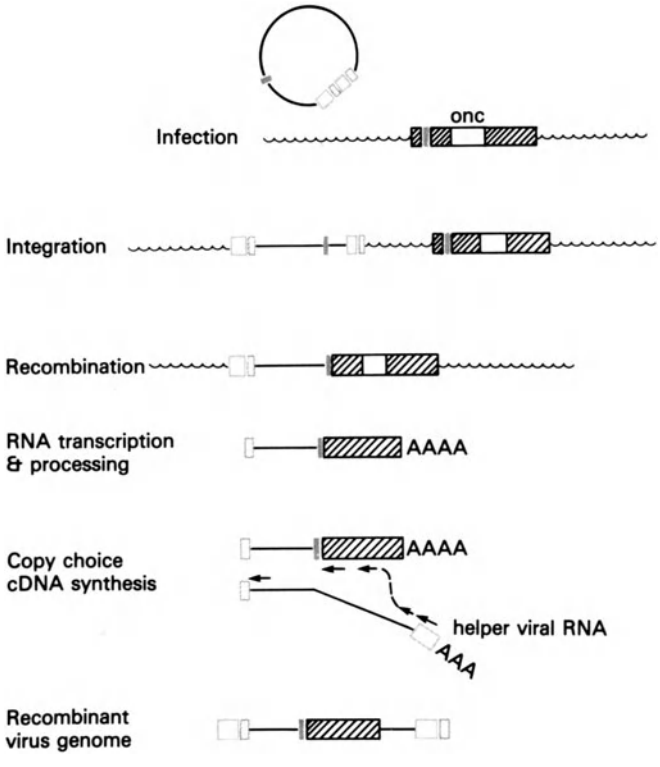

Fig. 5. Model for generation of acutely transforming retroviruses. (a) Infection: a circular helper virus genome shown here with two LTRs in the vicinity of a cellular *onc* gene. For simplicity, the cellular gene is shown to have two exons (*hatched area*) and one intron. The *small solid bar* represents homologous sequences in the helper virus and *c-onc*. *Wavy lines* are cellular sequences flanking *c-onc*. (b) The linearized helper provirus integrates upstream from *c-onc*. (c) Recombination between the homologous sequences deleting out the intermediary stretch (including the transcriptional stop signals of the virus genome). (d) Cotranscription of viral and cellular sequences and removal of the intron of *c-onc*. (e) Copy choice cDNA synthesis. (f) Generation of recombinant virus genome containing viral sequences flanking a cellular insert

intermediary sequences. The site of recombination may occur within a gene, an exon, or even a codon, as exemplified by *sis*. Transcription of the newly juxtaposed sequences results in a chimeric RNA molecule with viral and cellular sequences which is then processed to remove the introns of that part of the cellular gene. Simultaneous presence of complete viral and chimeric RNA probably held together by a dimer linkage at the 5′ end makes possible copy choice DNA synthesis, resulting in a recombinant DNA with viral sequences at both ends and a cellular sequence inserted. This DNA can then integrate into host chromosomes, and RNA transcripts from it can be efficiently packaged into virions. Some aspects of the latter steps of this model have been proposed by others [1, 7].

D. Expression of *onc* Gene Homologues in Human Cells

The identification of human *onc* gene homologues obviously raised the question of whether these are functional genes and whether they play a role in normal or neoplastic cell growth. We have examined a wide variety of human cells for the expression of *onc* gene homologues of Abelson murine leukemia virus (*abl*), avian myelocytomatosis virus (*myc*), avian myeloblastosis virus (*myb*), Harvey murine sarcoma virus (Ha-*ras*), simian sarcoma virus (*sis*), and feline sarcoma virus (*fes*). Molecularly cloned probes containing the *v-onc* sequences are labeled and hybridized to poly (A) containing RNA by the gel blotting technique described by Thomas [9].

I. Hematopoietic Cells

Our source of human hematopoietic cells includes fresh uncultered cells from normal individuals and leukemic patients as well as various cell lines of defined marker characteristics representing cells of myeloid, lymphoid, and erythroid lineages arrested at different stages of cell differentiation. The results [10, 11] are summarized in Table 1. Several points can be generalized from these studies: (1) There is no obvious difference between fresh and cultured cells of the same lineage, e.g., fresh AML cells and T-ALL cells behave as myeloblast cell lines (KG-1) and T-lymphoblast lines (CCRF-CEM, Molt-4) respectively. This argues for the validity of studying cell lines in these experiments. (2) The size(s) of mRNA for a given *onc* gene is the same in all human cells, and very similar (though not necessarily identical) to that of mRNA in other vertebrate cells. (3) The patterns of expression of different *onc* genes vary. Thus, each *c-onc* gene should be considered separate from the others. Specifically, the *abl* and Ha-*ras* genes are detectably expressed (one to five copies per cell) in all hematopoietic cells examined as multiple mRNA species. These genes are probably important for some basic cellular functions. The *myc* and *myb* genes code for single size transcripts of 2.7-kb and 4.5-kb respectively. However, the expression of *myb* is more restricted than *myc*. The *myc* gene is transcribed in all hematopoietic cells examined, including normal peripheral blood lymphocytes prior to or after stimulation with PHA. The only exception is terminally differentiated HL60 cells where *myc* transcription is turned off. The *myb* gene is expressed in the early precursor cells of lymphoid, myeloid, and erythroid lineages, but there is little or no expression relatively early in B-lymphoid cell differentiation, and late in T-cell or myeloid cell differentiation. Like *myc, myb* is transcribed in undifferentiated HL60 cells but not in HL60 cells induced to differentiate with either DMSO or retinoic acid. The *sis* and *fes* genes are not commonly transcribed in hematopoietic cells. There are two instances where enhanced transcription is observed: *myc* transcription in the promyelocytic cell line HL60 and *myb* transcription in leukemic T-lymphoblasts. However, it is premature to conclude that these enhanced expressions are disease related. Normal cells of equivalent lineages and stages of differentiation and/or more samples of similar disease types will be needed to clarify the correlation further.

II. Solid Tumors

A parallel study of human solid tumors and normal fibroblast cell lines has been carried

Table 1. Expression of *onc* genes in human hematopoietic cells [10, 11]

Cell type	Source	Stage of differentiation	mRNA species detected with					
			v-abl (kb) 7.2, 6.4 3.8, 2.0	*v-myc* (kb) 2.7	*v-myb* (kb) 4.5	*v*-H-*ras* (kb) 6.5 5.8, 1.5	*v-sis* (kb) 4.3	*v-fes* ?
Myeloid	KG-1, fresh AML	Myeloblast	+ +	+ +	+ +	+	–	–
	HL60	Promyelocyte	+ +	+ + + +	+ +	+	–	–
	HL60 + DMSO, RA	Granulocyte	+ +	±	–	+	–	–
Erythroid	K562	(Immature erythroid precursor)	+ +	+ +	+ +	+	–	–
Lymphoid T cells:	CEM, Molt-4 fresh ALL	Immature T cell	+ +	+ +	+ + +	+	–	–
	HUT78	Mature T cell	+ +	+ +	–	+	–	–
	HUT102	Mature T cell	+ +	+ +	–	+	+	–
B cells:	Raji, Daudi	Burkitt lymphoma	+ +	+ +	–	+	–	–
	NC37, CRB	EBV transformed	+ +	+ +	–	+	–	–
Normal peripheral lymphocytes			NT	+ +	–	NT	NT	NT
Normal peripheral lymphocytes + PHA			NT	+ +	–	NT	NT	NT

NT, not tested

	myb	*myc*	*sis*[a]	*abl*	Ha-*ras*
Sarcomas					
Rhabdomyo-	–	+	+	+	+
Osteogenic	–	+	+	+	+
Fibro-	–	+	+	+	+
Synovial	–	+	+	+	+
Carcinomas					
Skin	–	+	–	+	+
Lung	–	+	–	+	+
GI	–	+	–	+	+
Renal	–	+	–	+	+
Bladder	–	+	–	+	+
Ovarian	–	+	–	+	+
Others					
Melanoma	–	+	–	+	+
Glioblastoma	–	+	+	+	+
Teratoma	–	+	–	+	+
Normal fibroblast	–	+	–	+	+

Table 2. Expression of *onc* gene homologues in human solid tumor cell lines [4]

[a] *sis* is expressed in a high percentage, but not all, of the tumors indicated

out in collaboration with others and in particular Aaronson and colleagues [4]. The results as summarized in Table 2 reinforced the universality of expression of *abl* and Ha-*ras* genes. The *myb* gene is not expressed in these cells and may be specifically involved in hematopoietic cell differentiation. Of interest is the finding that *c-sis* is frequently expressed at moderate to high levels in sarcomas and glioblastomas but not in any melanomas, carcinomas, or normal fibroblast cell lines. Therefore, expression of this gene shows the greatest correlation with specific types of neoplasias.

E. Summary and Conclusion

Viral transforming (*v-onc*) genes are derived from cellular (*c-onc*) genes that are highly conserved among vertebrates. Comparative studies of *v-onc* and *c-onc* genes have shed some light on the mechanism leading to formation of the transforming viruses. A specific example of the *sis* gene is presented here for illustration. Studies on the expression of six *c-onc* genes in human cells revealed at least three categories of *onc* genes: (a) those that are universally expressed and probably are important in basic cellular functions, (b) those that are not detectably expressed in the cells examined and may have very transient expression in development, and (c) those that are only expressed in specific cell types and may be important in tissue differentiation. Our studies do not show conclusively a role of these *onc* genes in human neoplasias.

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The Biology of an Oncogene, Based Upon Studies on Neoplasia in *Xiphophorus*

F. Anders*

Xiphophorus, including swordtails and platyfish, is a genus of small viviparous freshwater fish from Central America that serves increasingly as a laboratory animal [1]. For 25 years we have used *Xiphophorus* for studies on neoplasia, which can easily be induced in hybrids between descendants of different provenance [2]. Although neoplasia of these animals is rather well understood in terms of formal genetics, the molecular basis of this phenomenon was extremely resistant to any elucidation. Recently a promising approach to the study of neoplasia in *Xiphophorus* at the molecular level has been undertaken in a cooperative work of the laboratories of H. Bauer (Institut für Virologie, Giessen), W. and H. Kersten (Institut für Physiologische Chemie, Erlangen), S. Nishimura (National Cancer Center Research Institute, Tokyo), and our laboratories. The present review will trace some steps of our studies that led to the detection of a cellular oncogene and a prominent regulatory gene.

A. The Taxonomic Groups of *Xiphophorus*

The genus *Xiphophorus* lives in genetically isolated populations in brooks, rivers, lakes, ponds, and pools, and has evolved into innumerable genotypically and phenotypically distinguishable groups [1]. Based on certain morphological and ecological characters, 17 of these groups have been listed as species [3].

All individuals of this genus, however, can be hybridized in the laboratory without difficulty, and all hybrids are fertile. This, together with the findings on the conformity of genome organization [4], the low degree of enzyme polymorphism [5, 6], and the normal chromosome pairing during meiosis in the hybrids [7], led to the conclusion that the taxonomic differences between these groups of *Xiphophorus* are not at the species level, but at the level of elementary local populations as well as ecological and geographical races.

B. Insusceptibility to Neoplasia in Wild Populations and Their Purebred Laboratory Descendants

Tens of thousands of individuals from different wild populations of *Xiphophorus* have been collected by several investigators (see [2]), but no tumors were detected. In the progeny of the wild populations, which in the case of *X. helleri* from Rio Lancetilla and *X. maculatus* from Rio Jamapa have been bred in the laboratory since 1939 (about 80 and 120 generations, respectively), no tumors occurred. About 10,000 specimens of purebred descendants of the wild populations have been treated with powerful carcinogens such as benzo(a)pyrene, N-methyl-N-nitrosourea (MNU), and X-rays, but none developed neoplasia. These animals are highly insusceptible to neoplasia (Table 1, first part).

* This contribution appears also in the 33rd Mosbach Colloquium: "The Biochemistry and Differentiation of Morphogenesis", also published by Springer

	No. of survivors		No. of neoplasms	
	MNU	X-rays	MNU	X-rays
Purebred				
X. maculatus	410	3405	0	0
X. variatus	ca 100	ca 500	0	0
X. xiphidium	ca 100	ca 100	0	0
X. helleri	415	ca 2000	0	0
X. cortezi	ca 100	ca 100	0	0
	ca 7200		0	
Hybrids				
F_1	470	ca 1000	18 (4%)	0
$F_2 - F_{24}$; BC_n	8258	3587	826 (10%)	163 (5%)
	ca 13,500		1007 (7.5%)	

Table 1. Neoplasia in *Xiphophorus* 1 year after treatment with MNU (10^{-3} M; four times for 1 h at 2-week intervals) and X-rays (1000 R; three times for 45 min at 6-week intervals)

C. Susceptibility to Neoplasia in Laboratory Hybrid Populations

In contrast to the animals from purebred wild populations, animals from laboratory hybrid populations derived from crosses between the purebred descendants of wild populations may be susceptible to neoplasia. Following treatment with carcinogens, depending on the wild populations used for hybridization, about 1%–4% of the F_1-hybrids develop neoplasia. Tumor incidence increases in the second hybrid generation (F_2) up to about 10% and remains stable in the succeeding generations, which have been tested up to F_{24} (Table 1, second part).

D. Classification of Neoplasms

As compiled from the results of several investigators in our laboratories [8] 805 of 10,195 (8%) hybrids which survived treatment with MNU and X-rays developed a large variety of different neoplasms. Most of the neoplasms were classified as neurogenic and mesenchymal, with melanoma, neuroblastoma, and fibrosarcoma being the predominant types. Epithelial neoplasms were less frequent but comprised the largest diversity (Table 2).

E. Assignment of Cancer Susceptibility to Chromosomes

To study what may be the crucial differences between the fish that were insusceptible and those that were susceptible to cancer, we attempted to assign the carcinogen-triggered neoplasms to chromosomes. For this study 65 defined genotypes of *X. maculatus, X. xiphidium, X. variatus, X. cortezi, X. helleri,* and their hybrids were employed [9, 10]. The genotypes exhibit, or lack, specific color patterns or enzyme markers which are due to the expression of specific genes, of which each is located on a different chromosome. We used mainly backcrosses, which were selectively bred for a specific phenotypic marker, and thereby for a specific chromosome. Such backcrosses segregate into 50% animals carrying the marker chromosome, and 50% lacking this chromosome.

Neoplasia could be assigned specifically to many different chromosomes. In the example shown in the scheme of Fig. 1 almost exclusively the backcross (BC) segregants exhibiting the stripes inherited from *X. maculatus* were susceptible. They developed melanoma, neuroblastoma, ephithelioma, and fibrosarcoma. Some of these hybrids developed several tumors of different types. This is not to say that all fish exhibiting the stripes develop neoplasia; but almost all neoplasms develop in those animals that belong to the group of

Table 2. Neoplasms induced in $F_2 - F_{24}$ and BC_n generations (MNU: 6608 survivors; X-rays: 3587 survivors; total: 10,195)

Type of neoplasm	No. of neoplasms			Incidence (%) based on total No. of survivors	
	MNU	X-rays		MNU	X-rays
Neurogenic					
Melanoma (benign)	135	93		2.12	2.6
Melanoma (malignant)	138	34	491	2.09	0.95
Neuroblastoma	84	7		1.27	0.2
Epithelial					
Squamous cell carcinoma	6	0		0.09	0
Epithelioma	19	6		0.28	0.17
Carcinoma (low-differentiated)	3	4		0.05	0.11
Carcinoma (high-differentiated)	2	5		0.03	0.14
Adenocarcinoma (kidney)	8	2	78	0.12	0.05
Adenocarcinoma (thyroid)	2	3		0.03	0.08
Papilloma	9	0		0.14	0
Hepatoma	5	1		0.07	0.03
Acanthoma	3	0		0.04	0
Mesenchymal					
Fibrosarcoma	190	6		2.87	0.17
Rhabdomyosarcoma	33	2	236	0.5	0.05
Lymphosarcoma	1	0		0.01	0
Reticulosarcoma	4	0		0.06	0
Total	642	163	805		

805 of 10,195 (7.9%) hybrids developed neoplasia; 92% of the hybrids were sufficiently protected

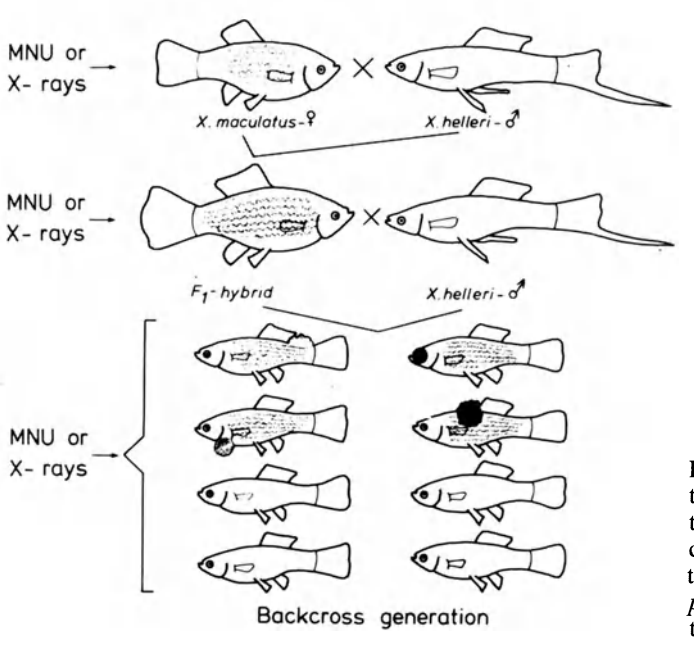

Fig. 1. Crossing scheme showing the assignment of carcinogen-triggered neoplasms to backcross segregants exhibiting stripes that are inherited from *Xiphophorus maculatus*. For details see text

BC segregants exhibiting stripes. Thus, the susceptibility to develop different kinds of neoplasms apparently depends on the marker chromosome which is responsible for the stripes. There are other examples in which susceptibility to neurogenic and epithelial neoplasia could be assigned to a certain chromosome while susceptibility to neoplasms of mesenchymal origin depends on a different chromosome [10]. Other examples have shown that susceptibility to develop melanoma may depend on a Y-chromosome, an X-chromosome, or an autosome. For all backcross hybrids tested so far, susceptibility to develop neoplasia is apparently chromosome specific.

F. Assignmentof Cancer Susceptibility to Oncogenes and Regulatory Genes

Information about the genes underlying susceptibility to carcinogen-triggered neoplasia comes from the analysis of the hereditary trait of certain "spontaneously" developing melanomas, pterinophoromas, neuroblastomas, thyroid carcinomas, kidney adenocarcinomas, and reticulosarcomas in hybrids. These neoplasms are very rare compared with the carcinogen-triggered tumors and therefore have been considered as a curiosity by many cancer researchers. They can, however, be produced at the will of the experimenter and have therefore been studied by many investigators [1]. They have contributed many important facts for developing ideas about how other animals, including humans, might inherit cancer [11, 12].

In the following chapters we shall mainly deal with the genes involved in melanoma development because their phenotypic effect, due to the enlarged shape and heavy pigmentation of the transformed cells, can easily be observed without killing the fish. Even a single transformed cell can be distinguished from a regular pigment cell.

Crosses of a spotted *X. maculatus* (platyfish) with a nonspotted *X. helleri* (swordtail) result in F_1-hybrids that develop benign melanoma instead of spots (Figs. 2, 3). Backcrosses of the F_1-hybrids using the swordtail as the recurrent parent result in offspring (BC_1), 50% of which exhibit neither spots nor melanoma while 25% develop benign melanoma (like the F_1), and 25% develop malginant melanoma. Further backcrosses of the fish (not shown in Fig. 3) carrying benign melanoma with the swordtail result in a BC_2 that exhibits the same segregation as the BC_1. The same applies for further backcrosses. Backcrossing of the fish carrying malignant melanoma with the swordtail results in a BC_2 in which 50% of the animals do not develop melanoma, while the remaining 50% develop malignant melanoma. In contrast, backcrossing of the melanoma-bearing hybrids using the platyfish as the recurrent parent results in a gradual suppression and finally disappearance of neoplasia in the succeeding generations.

These results, with the inclusion of cytogenetic findings, were interpreted as follows [2]: The spots and their corresponding genes are a specific accessory of the platyfish. The melanomas of the hybrids are closely related to the spots which actually are extreme benign melanomas. On the other hand the swordtail lacks both the corresponding spots and genes (Fig. 3).

The genetic information for neoplastic transformation of pigment cells in encoded in a "tumor gene" (*Tu*) which is inherited by the platyfish. About 70 crossovers, deletions, duplications, and translocations show that *Tu* is located at the end of the X-chromosome and is under control of linked and nonlinked regulatory genes [13]. In the platyfish used in this experiment a "major" pigment cell-specific regulatory gene (*R*) linked to *Tu* as well as two "minor" regulatory genes, which compartment-specifically suppress melanoma formation in the dorsal fin (R_{Df}) and the posterior part of the body (R_{Pp}), are mutated to R', R_{Df}', and R_{Pp}', respectively, and can no longer suppress *Tu*. Evidence for this comes from the appearance of some transformed pigment cells in the dorsal fin and in the skin of the posterior part of the body. The regulatory gene that actually suppresses tumor formation in the platyfish used in this experiment is the homozygous nonlinked "differentiation gene" (*Diff*) which can easily be detected by the esterase marker (*Est-1*) closely linked to *Diff* [6, 14, 15]. Further regulatory genes also present in the system are not taken into consideration.

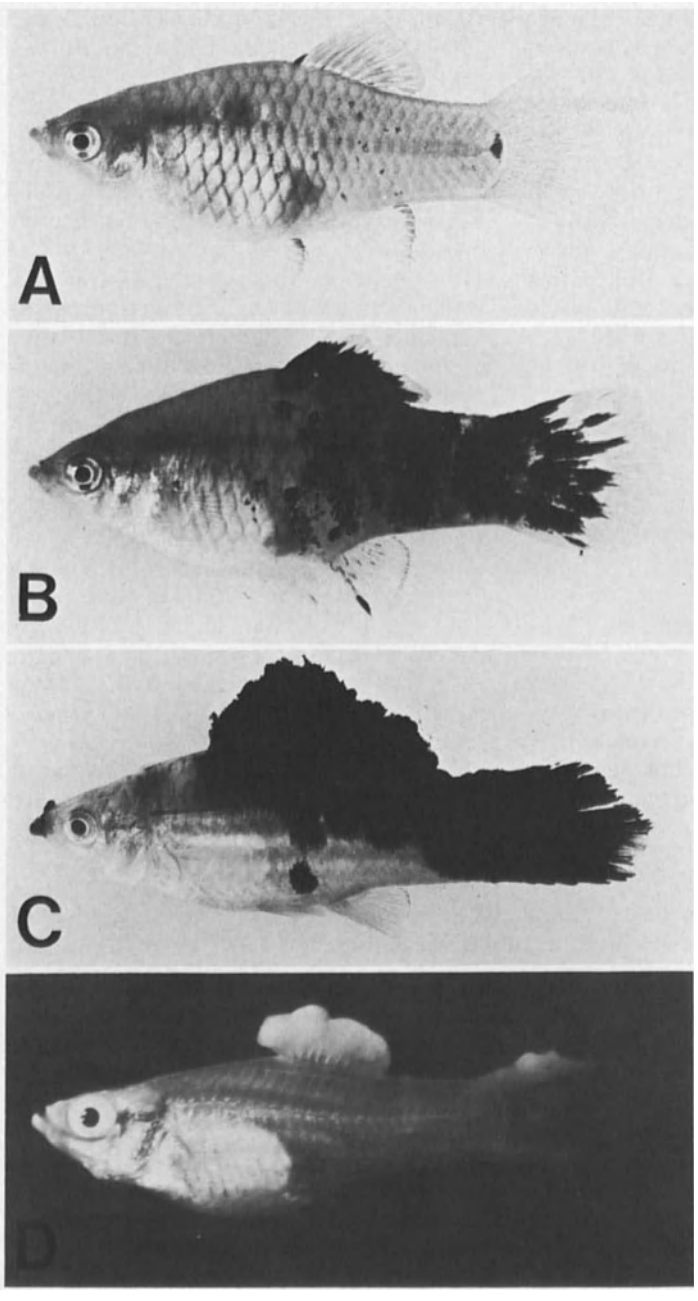

Fig. 2 A–D. Spots and melanomas in *Xiphophorus.* **A** *X. maculatus* from Rio Jamapa (Mexico); "spotted dorsal fin" (mutation R_{Df}) and "spotted posterior part" of the body (mutation R_{Pp}). **B** F_1-hybrid between *X. maculatus* **A** and *X. helleri* from Rio Lacetilla (Honduras) exhibiting benign melanoma instead of spots. **C** BC-hybrid **B** with *X. helleri* as the recurrent parent exhibiting malignant melanoma instead of spots. **D** Albino BC-hybrid exhibing malignant amelanotic melanoma. **A–C** correspond to the respective schematic drawings shown in Fig. 3

Following crossings and backcrossings using the swordtail as the recurrent parent, the chromosomes of the platyfish are replaced by the homologous chromosomes of the swordtail, resulting in the gradual disintegration of the regulating gene system for *Tu*.

In contrast, following backcrossings of the melanoma-bearing hybrids with the platyfish as the recurrent parent, the chromosomes carrying regulatory genes are reintroduced into the descendants. This results in a reconstruction of the original regulating gene system that suppresses the activity of *Tu*.

"Spontaneous" development of melanoma as well as its suppression following the appropriate crossing procedures was found in several experimental hybrid populations derived from different purebred populations of different geographical or ecological origin.

In order to disclose the genetic basis for the bulk of neoplasms that develop following treatment with a carcinogen, we have modified the experiment shown in Fig. 3 to the experiment shown in Fig. 4: The *R′ R_{Df}′ R_{Pp}′ Tu* chromosome was replaced by the *R R_{Df} R_{Bs}′ Tu* chromosome, the "major" *R* of which is nonmutated and active. Since this *R* is inherited along with *Tu*, neoplasia does not develop spontaneously in the hybrids. Following treatment with carcinogens, those hybrids carrying the *R-Tu* chromosome but lacking *Diff* (determined by the esterase) are highly susceptible to neoplasia. In this case development of neoplasia requires only impairment, or

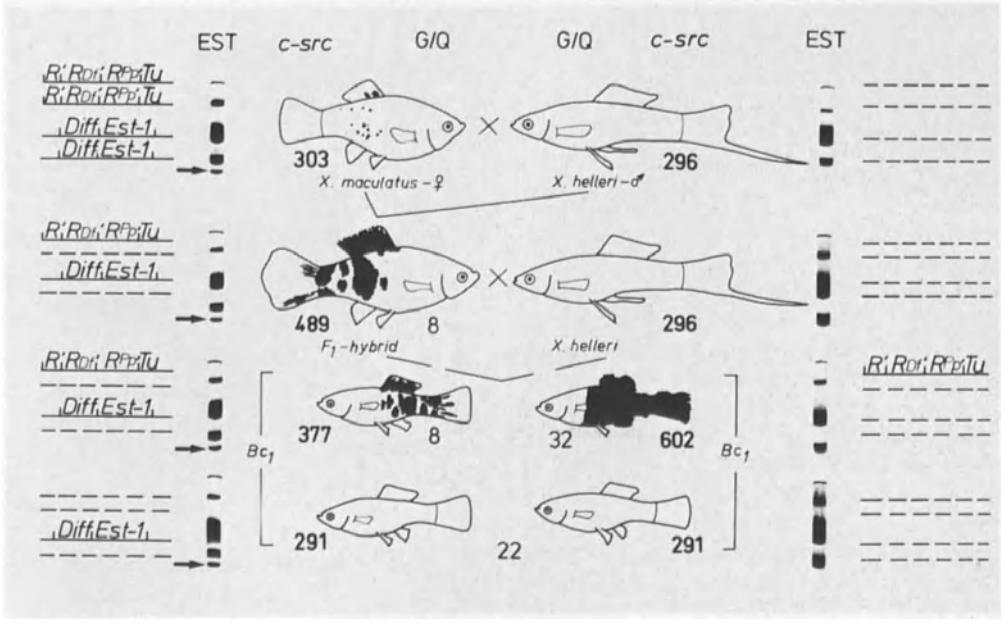

Fig. 3. Crossing scheme which displays the genetic conditions for the "spontaneous" development of spots (Fig. 2A), benign melanoma (Fig. 2B), and malignant melanoma (Fig. 2C). —— chromosomes of *X. maculatus;* ––– chromosomes of *X. helleri; Tu,* tumor gene; R_{Pp}′ and R_{Df}′, impaired regulatory genes controlling *Tu* in the compartments of the posterior part of the body (Pp) and the dorsal fin (Df); R′, impaired regulatory gene specific to pigment cells but nonspecific to the compartments; *Diff*, regulatory gene controlling differentiation of neoplastically transformed cells; *Est-1*, locus for esterase – 1 of *X. maculatus. EST,* esterases (polyacrylamide gel electrophoresis from homogenates of the eye); note linkage of *Diff* and *Est-1* (see *arrows*). *c-src,* $pp60^{c\text{-}src}$ kinase activity (cpm/mg protein; 53K; see Fig. 11); note basic and excessive activity, and correlation between *c-src* expression and *Tu* expression. G/Q, ^{3}H-guanine incorporation in position 34 (anticodon) of tRNAs for Tyr, Asn, Asp, and His (pmol/A_{260}; see Fig. 15); note low incorporation in tumors of *Diff* animals, indicating high Q content, and high incorporation in the melanoma of *Diff*-lacking animals, indicating low Q content (combined from data of [2, 6, 13, 14, 30, 31, 34, 40, 46]). For details see text

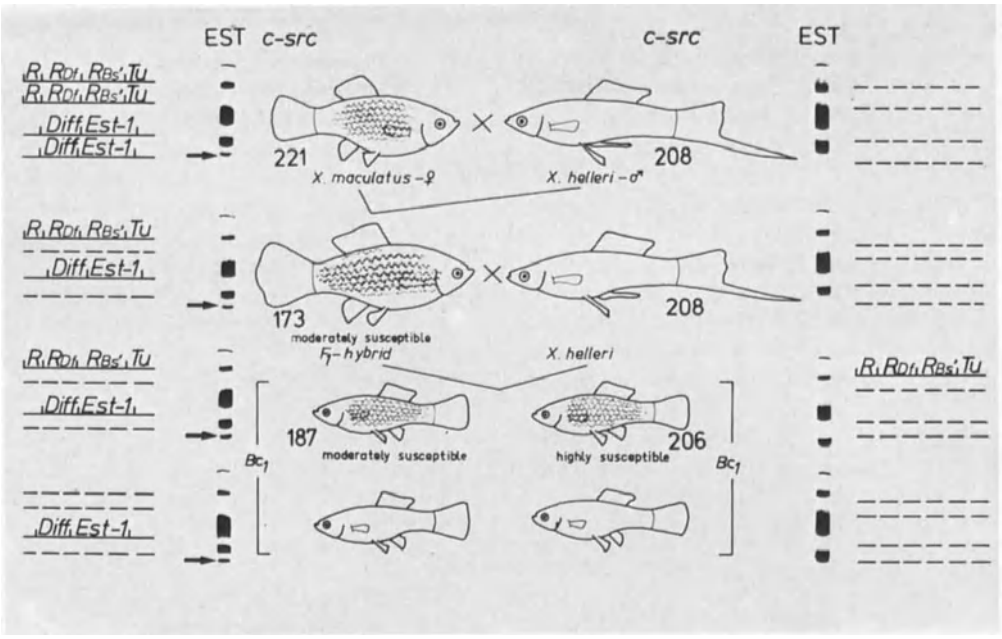

Fig. 4. Crossing scheme displaying the genetic conditions for susceptibility to carcinogen-dependent neoplasia. The highly susceptible genotype is highly sensitive to the carcinogenic (mutagenic) trigger. Abbreviations according to Fig. 3 R_{Bs}, impaired the regulatory gene controlling *Tu* in the compartment of the entire side of the body (Bs) (combined from data of [6, 16, 31, 34]). For details see text

deletion, of a single *R* gene by the carcinogen in a somatic cell [16].

In conclusion, the regulating gene systems confidently suppressing *Tu* in the nonhybrids become disorganized if chromosomes derived from different populations are combined in the hybrids by the experimenter. This implies that, in purebred fish, the *Tu* and its sets of regulatory genes are population-specifically coadapted by natural selection. In any case the genetic information coding for neoplastic transformation can be traced to a *Tu* which is present in the different cell types and is normally under control of population-specific and cell-type-specific polygenic systems of linked and nonlinked regulatory genes, which suppress the development of the various types of potential neoplasms (Fig. 5). According to the formal assignment of different neoplasms to a particular chromosome, the particular *Tu* that codes for neoplastic transformation of pigment cells may also code for transformation of cells of mesenchymal and epithelial origin (see Figs. 1, 5).

G. Enhancement of Melanin Pigmentation in Melanoma as an Epiphenomenon of *Tu* Expression

To study the relationship between melanoma formation and melanin synthesis [17, 18] we separated both processes by introgression of an albino gene into melanoma-bearing hybrids. The result was albinos which developed melanomas that completely lacked the melanin (Fig. 2D). This indicates that enhanced melanin pigmentation of melanomas is an epiphenomenon of neoplastic transformation of pigment cells exerted by *Tu*.

H. The Competent Cells for the Activity of *Tu* in the Pigment Cell System

The precursors of the melanin-producing pigment cells of *Xiphophorus,* like those of other vertebrates, originate from the neural crest, and migrate to their final destination (see Fig. 5). They divide and undergo dif-

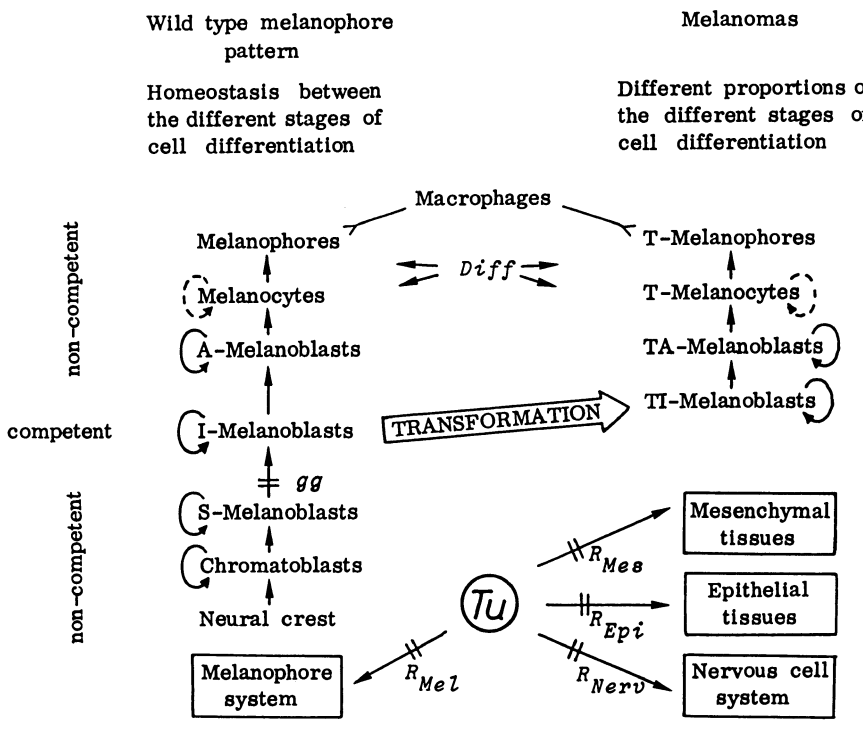

Fig. 5. Schematic presentation of the differentiation of normal and neoplastically transformed pigment cells. Only the intermediate melanoblasts (I-melanoblasts) are competent for transformation. *Tu,* tumor gene; R_{Mel}, pigment cell-specific regulatory genes for control of *Tu; gg,* homozygous "golden" mutation that blocks pigment cell differentiation; R_{Nerv}, R_{Epi}, R_{Mes}, regulatory genes that control *Tu* in the nervus cell system, the epithelial tissues and the mesenchymal tissues, respectively; *Diff,* regulatory gene which supports cell differentiation. Macrophages attack only the terminally differentiated pigment cells (both normal and transformed). (According to a scheme in [2], modified.) For details see text

ferentiation through the stages of chromatoblasts, stem(S)-melanoblasts, intermediate(I)-melanoblasts, advanced(A)-melanoblasts, melanocytes, and, finally, differentiate to melanophores, which are incapable of dividing. At a certain age the melanophores are removed by macrophages. Supply comes from S-melanoblasts [2, 19, 20].

A-melanoblasts, melanocytes, and melanophores have never been observed to undergo neoplastic transformation. Thus, these cells appear to be noncompetent for the *Tu* activity. On the other hand, in genotypes carrying a mutation that arrests differentiation at the stage of S-melanoblasts, melanomas cannot develop until exogenous promoters push differentiation of S-melanoblasts to the stage of I-melanoblasts [21]. These studies show that also the neural crest cells, chromatoblasts, and S-melanoblasts are noncompetent. We therefore conclude that the only stage of differentiation in which the pigment cells are competent for the transforming activity of *Tu* is the stage of I-melanoblasts. These cells become transformed to TI-melanoblasts. We assume that the principle of competence of a cell to the transforming activity of *Tu* applies also for other kinds of neoplasms.

It has not been possible to show so far whether *Tu* is still active in the transformed cells (T cells) for the maintenance of the neoplastic state. In any case the TI-melanoblasts continue to differentiate to TA-melanoblasts, T-melanocytes and, finally, to T-melanophores that are incapable of dividing. This process corresponds to differentiation of the nontransformed pigment cells. It is not under the control of *Tu* but under the control of the

differentiation gene *Diff*. This, furthermore, implies that *Tu* does not specify the degree of malignancy of the melanoma (see later).

I. The Genuine Effect of *Tu* in the Pigment Cell System

Information about the genuine effect of *Tu* comes from a balanced laboratory stock carrying a lethal *Tu* translocation that originated according to Fig. 6. *Tu* from an X-chromosome of *X. maculatus* becomes translocated to an autosome of *X. helleri* and, in its new position, is not under control of its former linked regulatory genes (*R*, R_{Df}, etc.). The progeny of this stock segregates into 50% carrying the nonlinked *Diff* which survive, while the corresponding 50% lacking *Diff* is lethal. As a consequence of the Mendelian inheritance of the *Tu* translocation through the germ line, and the lack of *Diff*, *Tu* becomes active in the developing progeny as soon as the pigment cell precursors differentiate to the competent I-melanoblasts.

This process starts in the 5-day-old embryos. Some time later some single dividing T-melanocytes appear at the peduncle of the tail fin of the embryo (Fig. 7). Neoplastic transformation continues in all areas of the developing embryo, where a pigment cell precursor becomes competent, thus building the lethal "whole body melanoma". This reflects the genuine effect of the completely derepressed *Tu* on the pigment cell system [13].

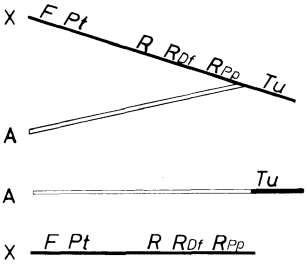

Fig. 6. Translocation of the tumor gene (*Tu*) from the X-chromosome (X) of *X. maculatus* to an autosome (A) of *X. helleri*. Note separaion of *Tu* from its linked regulatory genes (*R*, R_{Df}, R_{Pp}; see Fig. 3). *F*, sex-determining region of the X-chromosome; *Pt*, pterinophore locus. For the phenotypic effect see Fig. 7

It is suggestive to assume that *Tu* might exert an essential function in the early embryo which is related to the neural crest and its derivatives. In normal embryogenesis this function becomes switched off or choked by the regulatory genes prior to the 5th day of embryonic life. If, however, the regulatory genes (i.e., the entire switch) are lacking, *Tu* continues to exert its early embryo-specific function, which as a process of misguided cellular development transforms the competent cells to the neoplastic state.

J. Indispensable and Accessory *Tu* Copies

In the sex chromosomes of the platyfish 30 deletions involving *Tu* have been characterized genetically, and some of the major deletions involving both the *Tu* and its linked regulatory genes, additionally to the genetic results, were cytologically observed [13, 22]. All deletions are nonlethal. Even the loss of a *Tu*-containing segment of the X-chromosome (one Giemsa band), in the homozygous condition in the female or in the hemizygous state in the male, apparently has no detectable effect on viability. This, together with the fact that the swordtail used in our crossing experiments populationspecifically lacks the *Tu* (see Figs. 3, 4), led us to the conclusion that the *Tu* so far considered is not essential but is an accessory to the fish [2]. This is not to say that the *Tu* has no normal function. One could, for instance, assume that additional copies of *Tu* that are indispensable to the fish are present in the autosomes and may compensate for the loss of the sex chromosome-linked *Tu* loci according to a gene dosage compensation mechanism which warrants normal functions. Support for the assumption of multiple copies of *Tu* per haploid genome comes from the following experiment. Platyfish, carrying the deletion of the Giemsa band that involves the accessory *Tu*, were crossed according to the procedure outlined in Figs. 3, 4, with the swordtail populationspecifically lacking the accessory *Tu*. No tumors developed in the hybrids. Following treatment of the backcross hybrids with MNU, however, melanomas developed which were specifically localized at the upper part of the tail

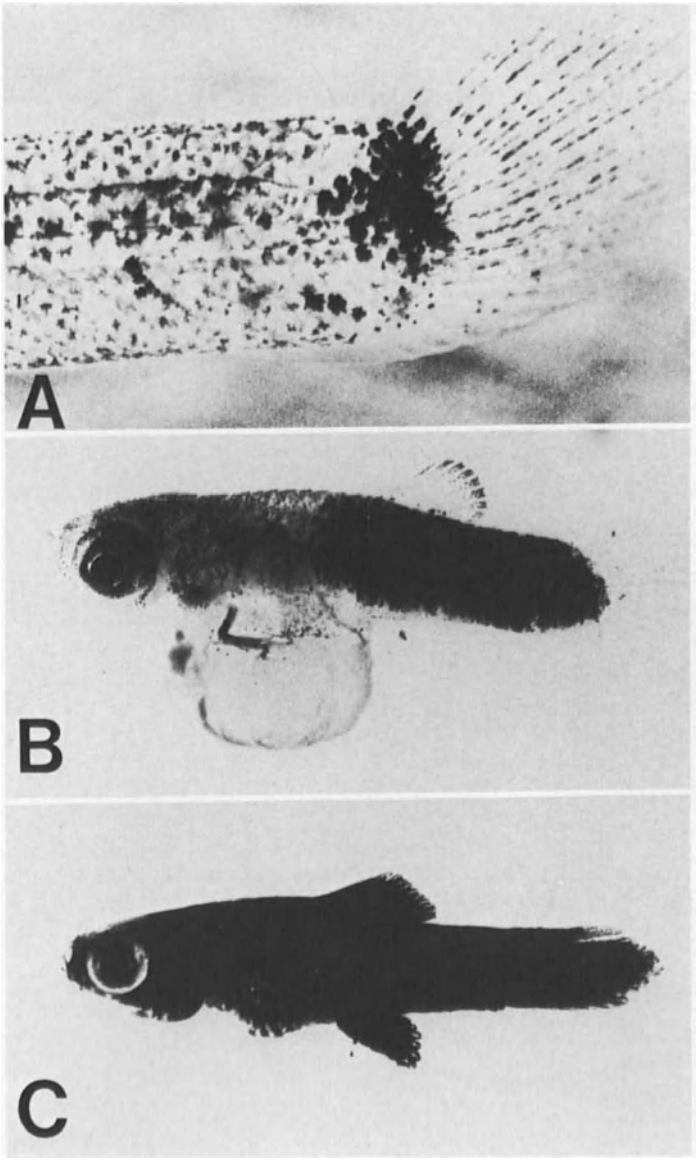

Fig. 7A–C. The genuine effect of the tumor gene *Tu* (corresponding to the scheme of translocation shown in Fig. 6). **A** Tail of a 10-day-old embryo (3 mm in length) exhibiting some T-melanocytes at the peduncle of the tail fin. **B** The same fish, 5 days later (4 mm in length). **C** Neonate of the same genotype (6 mm in length)

fin. These neoplasms could be assigned to an autosome. Thus it appears that the platyfish, besides the easily detectable accessory *Tu* copies contains additional ones that require more intricate experiments for their detection.

Our experience that all individuals of all groups of *Xiphophorus* can contribute to susceptibility to neoplasia in the hybrids suggests that all individuals contain *Tu* copies that are indispensable for the fish, and may contain accessory *Tu* copies. Up to ten accessory copies of the repressed *Tu* could be introduced into a laboratory stock by crossings. No effect on viability could be observed.

K. Oncogene Dosage Effect and Oncogene Dosage Compensation

Both the X-chromosome of *X. maculatus* containing *Tu* (the X-chromosome according to Fig. 3) and the X-chromosome of *X. maculatus,* having lost the Giemsa band carrying *Tu,* were introduced into the genome of *X. helleri* lacking the regulatory genes for the accessory *Tu.* X^{Tu} X^{Del} × X^{Tu} Y matings were accomplished. The segegating offspring had none, one, or two, respectively, accessory *Tu* copies and showed a clear-cut gene dosage effect (Fig. 8). If, however, the experiment was modified by using animals as recipients, having retained the nonlinked regulatory genes, we observed a clear-cut gene dosage compensation (Fig. 9). Dosage effect and dosage compensation of the accessory oncogene *Tu,* therefore, depends on the absence or presence, of the nonlinked regulatory genes. Oncogene dosage effect and oncogene dosage compensation has been observed in many experiments of this kind [23].

L. Transfer of Accessory *Tu* Copies by Injection of DNA

DNA from laboratory platyfish carrying several accessory copies of *Tu* (derepressed

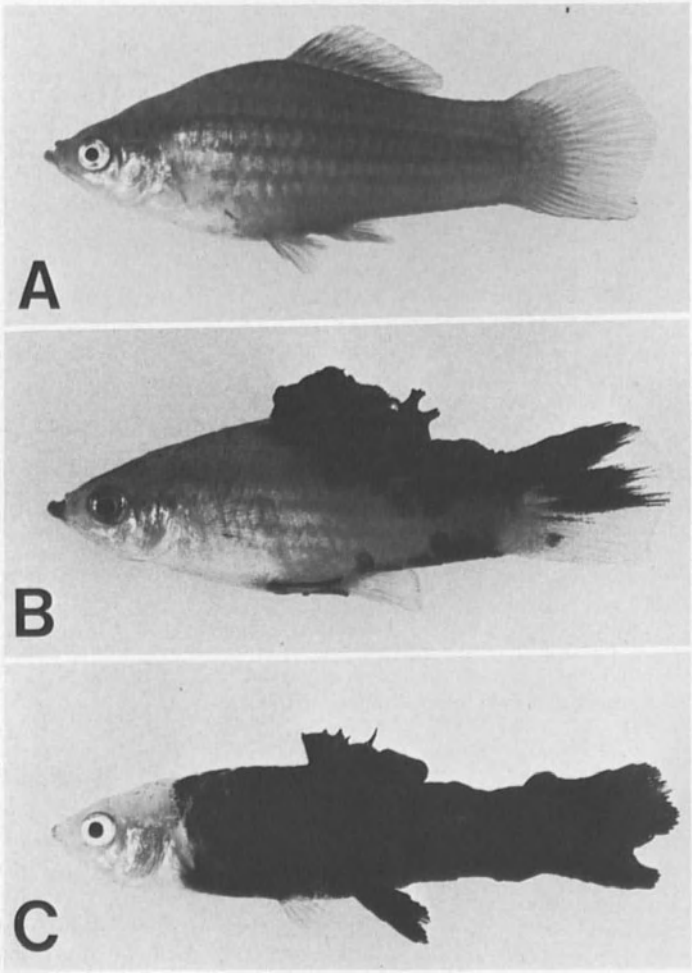

Fig. 8A–C. *Gene-dosage effect* of the (incompletely) *derepressed* tumor gene (*Tu*). **A** No *Tu* (not a single transformed pigment cell). **B** One dosage of *Tu* (melanoma formation). **C** Double dosage of *Tu* (double effect in melanoma formation). Compare with Fig. 9

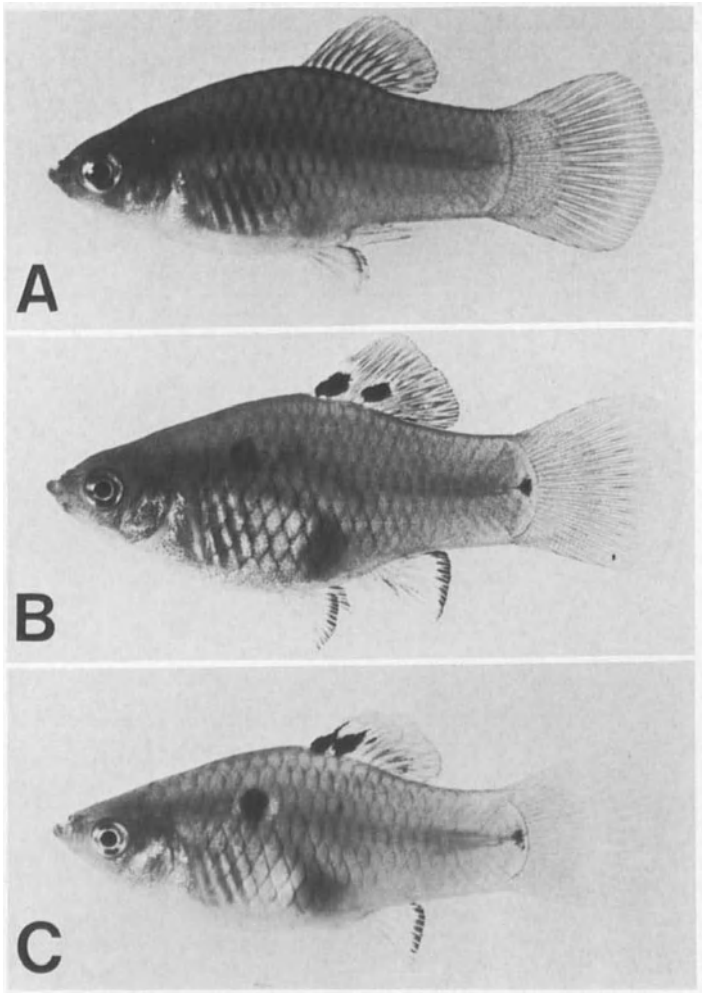

Fig. 9 A–C. *Gene dosage compensation* of the *repressed* tumor gene (*Tu*). **A** No *Tu* (not a single transformed pigment cell). **B** One dosage of *Tu* (spots consisting of transformed pigment cells; see dorsal fin). **C** Double dosage of *Tu* (effect shows no difference to that of one dosage). Compare with Fig. 8

as well as repressed) was injected into the neural crest region of early embryos of the swordtail which lacked both the accessory *Tu* copies and the regulatory genes (Fig. 10). The injected DNA may maintain its high molecular weight for about 2 h and thereafter becomes degraded to pieces which are too small to contain genetic information [24]. Following the successful uptake of *Tu* by an S-melanoblast of the embryo, this cell may later become competent to the *Tu* activity by differentiation to an I-melanoblast, which eventually may become neoplastically transformed to a TI-melanoblast. Additional proliferation of T cells amplifies the original transforming effect of *Tu*, and the result becomes visible as the transformed cells differentiate to the easily detectable colonies of T-melanocytes and T-melanophores [25].

Depending on the type of the *Tu* donor DNA (cotransfer of intact or damaged regulatory genes) the percentage of recipients showing T-melanocytes and T-melanophores ranged from 0.4% to almost 8% (total number of survivors tested in these experiments: 1390). Since the number of the target cells (pigment cell precursors) at the time of DNA injection has been estimated to be about 1000, the

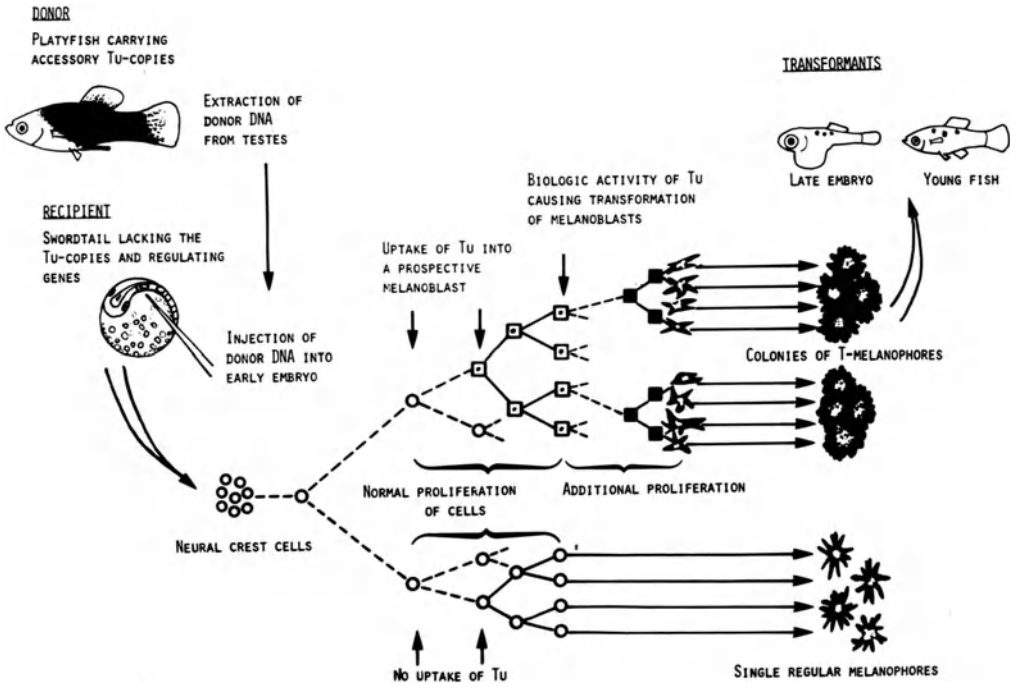

Fig. 10. Schematic presentation of the outcome of *Tu* transfer by injection of purified DNA (modified from a scheme in [25]). For details see text

frequency of the transforming event on the cellular basis is in the range of 10^{-5} [25]. This is the same order of transfection frequencies reported for cell culture systems [25–27].

M. The *c-src* Oncogene in the *Tu* Melanoma System

Several virological observations came to our knowledge which could be of interest for a molecular interpretation of the *Tu* gene: In chicken it was found that the oncogene *v-src* from Rous sarcoma virus (RSV) has a counterpart, *c-src*, in the noninfected cells [28]. *C-src* or at least a similar gene was also found in mammals including mouse, calf, and humans [29]; and commercial DNA derived from salmon [29] obviously contains the same gene. There is, however, no convincing evidence to relate the cellular *src* or its gene product, a 60,000-dalton phosphoprotein with kinase activity ($pp60^{c\text{-}src}$), to neoplasia that depends on conditions other than virus infections (see discussions in [30, 31]). With this background we started the search for *c-src* in the genome of *Xiphophorus*.

C-src was detected in *Xiphophorus* by molecular hybridization of a *src*-specific probe from cloned *v-src* with DNA from fish [32]. To identify $pp60^{c\text{-}src}$, brains of the fish were labeled with ^{32}P-orthophosphate, and brain extracts were immunoprecipitated with antisera from RSV tumor bearing rabbis (TBR-serum) followed by polyacrylamide gel electrophoresis. The 60K protein detected in the gel has a tyrosine-specific kinase activity, and represents the $pp60^{c\text{-}src}$ [31].

The kinase activity was measured according to Fig. 11 [33] and then determined (see legends of Figs. 3, 11) in several tissues including skin, liver, spleen, testes, brain, and melanoma. Brain and melanoma always had the highest kinase activity. Genotype-specific differences in kinase activity showed an identical trend in both brain and melanoma [31]. To compare *c-src* expression in nontumorous and tumorous fish, kinase activity was mainly determined in the brains of these fish.

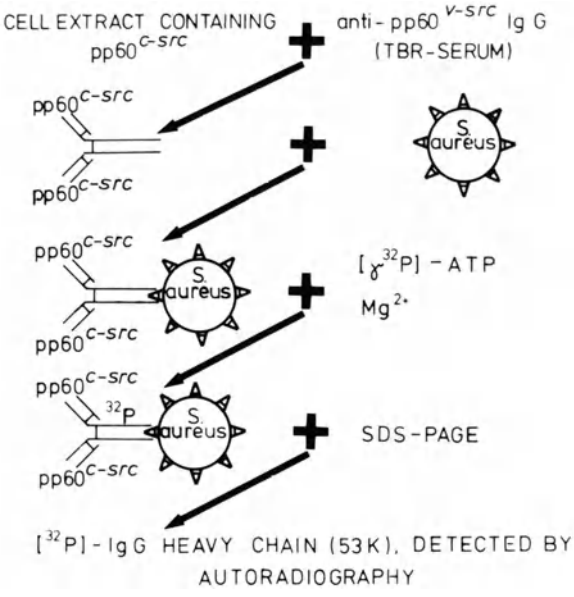

Fig. 11. Assay for pp60$^{c\text{-}src}$ kinase activity according to Collet and Erikson ([33], modified; see also [30, 31, 34])

To study the possible relation between neoplasia and *c-src* expression we manipulated neoplasia in *Xiphophorus* according to the three genetic experiments outlined in Figs. 3, 4 and 12 [34].

1. In the experiment recorded in Fig. 3 the purebred *X. maculatus* carrying two repressed copies of the accessory *Tu*, as well as the purebred *X. helleri* and the BC-hybrids lacking the *Tu*, display the same activity of *c-src* kinase. This activity appears to be the basic expression of *c-src*. In contrast, the melanoma-bearing hybrids which contain the derepressed *Tu* show an increase of *c-src* activity, with the malignant melanoma bearing BC-hybrids displaying the highest activities.

2. In the experiment recorded in Fig. 4 all purebred and hybrid animals, irrespective of the lack and the dosage of *Tu* but dependent upon the nontumorous state exerted either by several regulatory genes or by a linked *R* alone (see the highly susceptible genotype), display a uniform *c-src* activity which seems to represent the basic *c-src* expression, as in the purebred animals and *Tu* lacking hybrids in Fig. 3.

3. In littermates (Fig. 12) which are genetically identical except for the lack of the accessory *Tu* and the presence of one or two partially derepressed accessory *Tu* copies, *c-src* displays a kinase activity that increases stepwise in parallel to the lack and the dosage of *Tu*, which, in their turn, determine whether the animals will develop no tumors, slowly growing tumors, or fast-growing tumors. Table 3 shows additional experiments of the same kind that yielded similar results.

The main results of these experiments are that the nontumorous fish display a basic expression of *c-src* which in the tumorous fish may increase stepwise under two different conditions, namely (a) the stepwise derepression of an accessory *Tu*, and (b) the stepwise introduction of additional copies of a derepressed accessory *Tu*. Since the measurements were accomplished in the brains of the fish the increase of the activity of *c-src* is related di-

Table 3. pp60$^{c\text{-}src}$ associated kinase ativity in brain extracts specified by cpm per milligram soluble protein[a] in F_2-segregants (Data from [34])

Tu gene Complex[c]	Dosage of *Tu*[b]		
	–/–	*–/Tu*	*Tu/Tu*
Striped	90	200	390
Dabbed^{BR}	170	190	390
Dabbed^{RI}	200	260	1240

[a] Three to 8 brains per measurement
[b] One gel each
[c] Different gels each

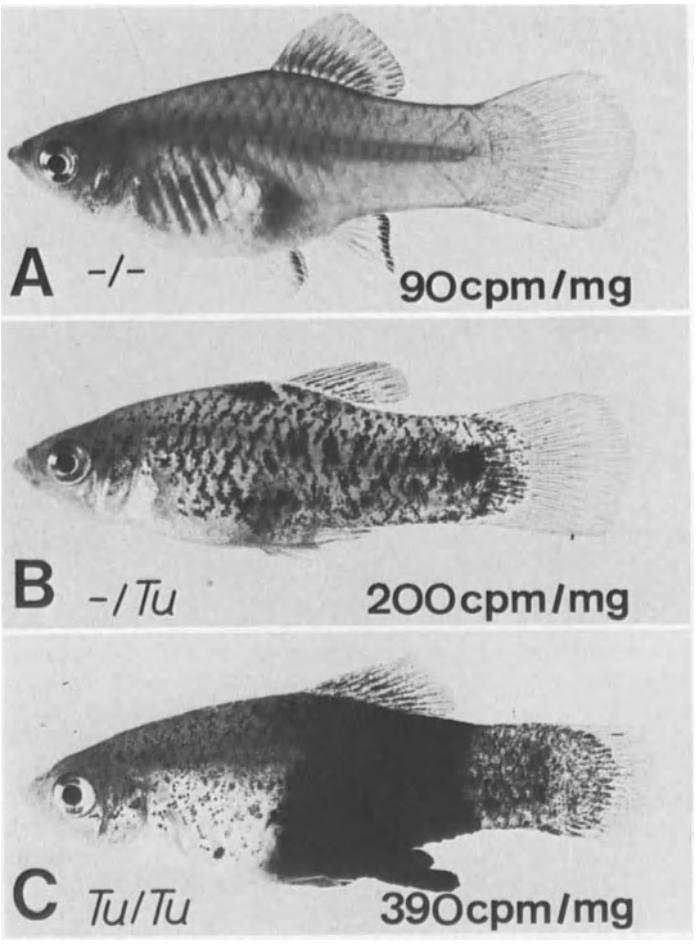

Fig. 12A–C. Correlation between gene dosage effect of *Tu* (phenotype of the tumor) and gene dosage effect of *c-src* (pp60$^{c\text{-}src}$ kinase activity) in littermates containing **A** no accessory *Tu*, **B** one dosage of *Tu*, **C** double dosage of *Tu*. The genetic backgrounds of the fish are identical. **A** *Tu* is deleted in the germ line. **B, C** The pigment cell-specific *R* linked to *Tu* is impaired by germ line mutation [13, 23]. Kinase data from [34]

rectly to the activity of *Tu*, and does not represent an epiphenomenon of melanoma formation, such as the elevated activity of many enzymes, [5, 6, 14, 15] enhanced melanin synthesis [18], certain chromosome aberrations [35], etc.

These findings suggest several possibilities for an interpretation of how *Tu* might be related to *c-src*: (a) *Tu* might be independent from *c-src*, and the correspondence between both *Tu* and *c-src* is due to linkage relationship. (b) *c-src* might represent a regulatory gene for *Tu* or *vice versa*. (c) *Tu* might consist of different oncogenes that are responsible for different kinds of neoplasia and *c-src* is one of these genes. (d) *Tu* might be identical to *c-src*, and this oncogene is capable of coding for a large variety of neoplasia. At present we cannot decide on a special interpretation. Additional data are required.

N. Distribution of *c-src* in the Animal Kingdom

The presence of *c-src* in the genome of different animals such as chicken, salmon [29], and *Xiphophorus* led to the more systematic search for this oncogene in additional taxonomic groups of animals. Firstly, different groups of *Xiphophorus* and different fish

Table 4. Expression of pp60$^{c\text{-}src}$ kinase in brain extracts of different fish species [31]

Xiphophorus helleri	from Belize River
X. helleri	from Rio Lancetilla
X. maculatus	from Belize River
X. maculatus	from Rio Jamapa
X. maculatus	from Rio Usumacinta
X. cortezi	
X. variatus	from Rio Coy
X. variatus	from Rio Panuco
Girardinus falcatus	
Girardinus metallicus	
Poecilia sphenops	
Belonesox belizanus	
Heterandria bimaculata	
Xenotoca eiseni	

genera more or less related to *Xiphophorus* were investigated. All fish tested (Table 4) show a pp60$^{c\text{-}src}$ kinase activity indicating that *c-src* must be present [31]. In addition, *c-src* was evidenced by its kinase activity in a large variety of metazoans other than fish, ranging from mammals to sponges, which, together with the results from other laboratories are listed in Table 5. *C-src* was not found in protozoa, algae, or higher plants [36].

The distribution of the cellular counterpart of the virl *v-src* brings about the idea that *c-src* might have evolved together with the multicellular organization of the animals, and that neoplasia might be a character that is closely related to this evolution.

O. The Regulatory Gene *Diff* in the *Tu* Melanoma System

The gene *Diff* is one of the most prominent regulatory genes known in the melanoma system of *Xiphophorus*. As shown in Fig. 3, benignancy and malignancy in the hybridization-conditioned melanomas depend upon the presence or absence of the chromosome carrying *Diff*. Biochemical markers for this chromosome, i.e., the esterase *Est-1* and the isozyme A of the glyceraldehyde-3-phosphate dehydrogenase [6] have confirmed that the *Diff*-carrying chromosome is derived from the platyfish, the source of accessory *Tu*.

P. The Major Characters of *Diff* Expressions

The clear-cut Mendelian segregation of benignancy and malignancy has provided the opportunity to study the basic differences between the benign and the malignant state of the melanomas (Fig. 13, Table 6). The cytological, fine structural, biochemical, and biological data suggest that *Diff* promotes the differentiation of T cells: If *Diff* is lacking, the majority of the melanoma cells persist in the stage of the poorly differentiated, continuously dividing TA-melanoblasts and T-melanocytes, and only few cells differentiate to the final stage of the T-melanophores. If, however, the *Diff* is present, the majority of the melanoma cells become terminally differentiated to T-melanophores, whereas only a few cells remain in the stage of TA-melanoblasts and T-melanocytes. The T-melanophores at a certain age are removed by macrophages ([14, 18, 20, 38], see also Fig. 5). This process is antagonistic to the permanent supply of melanoma cells from S-melanoblasts, and thus the melanoma is rendered benign [20, 21].

Table 5. *c-src* in eukaryotes (from [36])

Mammals	Bony fish	Cartilaginous fish	Insects
Humans [29]	Flat fish	Shark	Cockroach
Calf [29]	Sea robin		*Drosophila* [37]
Rat	Mackerel	Jawless fish	
Mouse	Roach	Lamprey	Sponges
	Gudgeon		Marine sponge
Birds	*Xiphophorus*	Acrania	Freshwater sponge
Chicken	Salmon [29]	*Amphioxus*	
Quail	Codfish		
	Cichlid		

Q. Diffusiveness of a *Diff* Dependent Product Involved in Differentiation

Transplantation experiments, including the composition of chimeras by fusion of parts of early embryos, have shown that pigment cell precursors present in the transplants taken from fish carrying *Tu* but lacking *Diff* (see Fig. 3) become incompletely differentiated and give rise to malignant melanoma if transplanted into embryos lacking *Tu* and *Diff*. If, however, the pigment cell precursors of the same genotype were transplanted into *Tu*-lacking embryos that contain the *Diff*, the cells of the developing melanoma become terminally differentiated and regain their distance regulation (Fig. 14). Thus the effect of *Diff* on the differentiation of the neoplastically transformed pigment cells can be traced to a diffusible substance [39]. The nature of this substance is unknown.

R. Modified tRNAs Involved in *Diff* Dependent Differentiation

There is considerable evidence for the involvement of tRNA containing modifications of the nucleotides in the process of cell differentiation in normal and neoplastic tissues [40]. Many studies were focused on a family of tRNAs including $tRNA^{Asn}$, $tRNA^{Asp}$, $tRNA^{His}$, and $tRNA^{Tyr}$ which may contain queuosine (Q) instead of guanosine (G) in the first position of the anticodon (position 34). Q is a hypermodified G. The more the differentiation progresses, the more G is replaced by Q in position 34 [40–46]. The method to estimate the G : Q ratio in a given popu-

Table 6. The Gene *Diff* in *Tu/-Xiphophorus* [2, 6, 14, 18, 20, 21, 38, 39, 40, 46]

Diff/–	–/–
Benign melanoma	Malignant melanoma
Differentiated	Poorly differentiated
Slow-growing	Fast-growing
Noninvasive	Invasive
Nonlethal	Lethal
Difficult to transplant	Easily transplantable
Difficult to promote	Promotion by testosterone, cAMP, corticotropin, BrdUrd, nutrient factors, etc.
Regression following testosterone treatment, etc.	No regression
No vascularization	Vascularization
Weak effect of external factors on growth rate	Drastic effect of external factors on growth rate (temperatue, salinity, cyclic Bt_2AMP, corticotropin, BrdUrd, etc.)
No effect of nutrient factors	Drastic effect of nutrient factors (amino acids)
Many macrophages	Few macrophages
T-melanophores prevail	TA-melanoblasts and T-melanocytes prevail
Endopolyploid and multinucleated	Diploid and uninucleated
Mature melanosomes	Immature melanosomes
Lack of ER and Golgi complexes	Well-developed ER and Golgi complexes
Low enzyme activities	High enzyme activities (tyrosinase, $LDH^{b}B4$, MDH^{c}, etc.)
Low rate of thymidine incorporation	High rate of thymidine incorporation and DNA synthesis
Low pteridine contents	High pteridine contents
First position of the anticodon of $tRNA^{Asp}$, $tRNA^{Asn}$, $tRNA^{Tyr}$, $tRNA^{His}$ contains predominantly queuosine	First position of the anticodon of $tRNA^{Asp}$, $tRNA^{Asn}$, $tRNA^{Tyr}$, $tRNA^{His}$ contains predominantly guanosine
Diff product is diffusible	No product

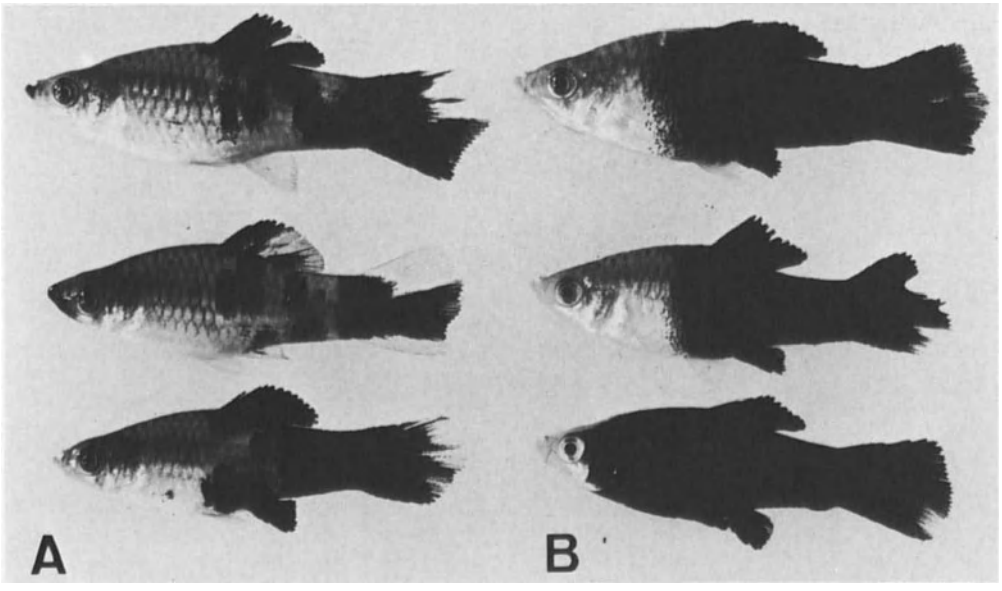

Fig. 13A, B. Segregants of littermates **A** containing one dosage of the differentiation gene (*Diff*) and **B** lacking *Diff*. Segregation of animals carrying benign and malignant melanoma is according to that of the backcross generation shown in the schematic drawings of Fig. 3

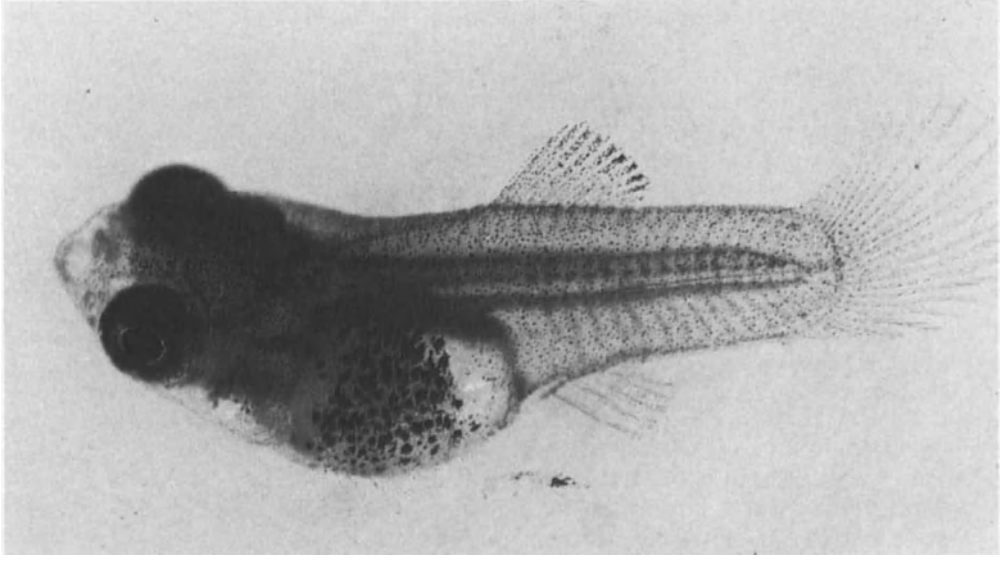

Fig. 14. Secondary chimera composed by transplantation of tissues containing precursor cells of malignant melanoma that originated from a young BC-hybrid containing *Tu* but lacking *Diff* (see malignant melanoma developing BC-segregant in Fig. 3), to a littermate lacking *Tu* but containing *Diff* (see the nontumorous BC-segregant at bottom left in Fig. 3). Note terminal differentiation and distance (density) regulation of the transformed cells of the transplant according to the *Diff* genotype of the host (from [39]). For details see text

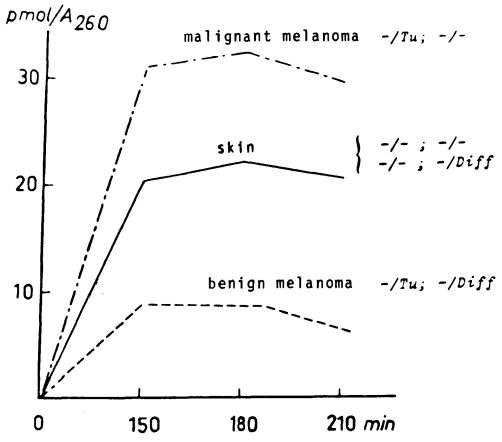

Fig. 15. Incorporation of ^{3}H-guanine in position 34 (anticodon) of tRNAs for Tyr, Asn, Asp, and His catalyzed by tRNA-guanine-transglycosylase (insertase) of *E. coli*. The melanomas (both malignant and benign) were derived from littermates of BC-hybrids according to Fig. 13 or Fig. 3, respectively. The skin was derived from both *Diff*-containing and *Diff*-lacking nontumorous BC-segregants according to Fig. 3. Compare with G/Q data shown in Fig. 3 (from [40, 46]). For details see text

lation of the tRNA family consists in following the replacement of quanine in position 34 by a labeled guanine exerted by a guanine-transglycosylase of *E. coli*.

We have measured the incorporation of ^{3}H-guanine in the tRNA for Asn, Asp, His, und Tyr in the malignant melanoma, the benign melanoma, and the skin of melanoma-free littermates. In addition, F_1-hybrids carrying benign melanomas were studied ([40, 46], Figs. 3, 15):

^{3}H-guanine incorporation is high if the tRNAs are prepared from malignant melanomas (predominantly poorly differentiated cells). In contrast, the incorporation is low if the tRNAs are derived from benign melanomas (predominantly well-differentiated cells). The tRNA family of the malignant melanomas, therefore, is G-rich, whereas the tRNA family of the benign melanomas is Q-rich.

^{3}H-guanine incorporation in the skin of nontumorous littermates is intermediate between those of the malignant and the benign melanomas. Since the nontumorous fish (like the tumorous ones) consist of individuals lacking and containing *Diff* in a 1 : 1 ratio, it is suggestive to assume that the intermediate data represent a mean value of ^{3}H-guanine incorporation in the *Diff*-containing group and in the *Diff*-lacking group. If this is correct the different G : Q ratios in the different melanomas are no epiphenomena of malignancy and benignancy, but are very closely related to the primary effect of *Diff* that in tumorous fish converts the malignant to the benign state.

S. Discussion

We have studied neoplasia of *Xiphophorus* at different levels of the biological organization including species, races, populations, generations, littermates, individuals, tissues, cells, genomes, chromosomes, and genes. In doing so we could trace neoplastic transformation to the activity of one or several copies of the oncogene *Tu* which shows a relation to a cellular counterpart of the transforming *src* oncogene of avian sarcoma virus, the *c-src* [30]. The normal function of *c-src* remained unknown. Since *c-src*, however, was also found in all individuals of all metazoans tested, and was not found in protozoans and plants, it might have some basic functions of life closely related to the multicellular organization of animals including humans [36].

In *Xiphophorus* it was shown further that the cellular oncogene is normally under control of systems of multiple regulatory genes corresponding to regulator genes of bacteria and phages. Some of the regulatory genes are located on other chromosomes than those bearing an oncogene. One of the most prominent regulatory genes appears to be responsible for terminal differentiation of the neoplastically transformed cells exerted via modification of nucleosides in the anticodon of certain tRNAs [40].

Interpopulational or interracial hybridization in preceding generations in *Xiphophorus* is the main event contributing to the disintegration of the regulatory gene system for the oncogene. Germ line mutations that may also disturb the regulatory gene systems are probably less important than hybridization because they are always rare, or may become repaired. Somatic mu-

tations and tumor promotion, which are the majority of carcinogenic triggers, may complete this disintegration. The majority of the neoplasms of *Xiphophorus* belongs to the types that are triggered by carcinogens or promoters on a competent genetic background like their counterparts in humans, which represent about 90% of all human neoplasms (see [8]).

The phenomenon of introducing susceptibility to neoplasia by means of hybridization is not limited to *Xiphophorus.* Many examples have been cited from the animal kingdom [8]. It appears that in animals from wild populations neoplasia is difficult to induce and "spontaneously" developing neoplasms are rare, while in animals of hybrid origin (domesticated and laboratory animals; naturally occurring and experimentally produced hybrids) neoplasia is easily inducible and the incidence of "spontaneously" developing neoplasms is high.

While we do not have hybridization in human beings comparable to hybridization of domesticated or laboratory animals such as fish and mice, it is suggestive to speculate how much effect hybridization may have had on the high tumor incidence observable in some of our highly developed nations. Such speculations are probably of little value in the fight against cancer, but in our search for the cause of human neoplasia they could help to realize the factors that make an individual susceptible to neoplasia and, therefore, sensitive to the carcinogens of our environment.

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The Transforming Gene of Avian Myeloblastosis Virus (AMV): Nucleotide Sequence Analysis and Identification of Its Translational Product

T. S. Papas, K. E. Rushlow, D. K. Watson, J. P. Bader, D. Ray, and E. P. Reddy

A. Abstract

The genome of the avian myeloblastosis virus (AMV) has undergone a sequence substitution in which a portion of the region normally coding for the *env* protein has been replaced by cellular sequences. We have determined the complete nucleotide sequence of this region. Examination of the AMV oncogenic sequence revealed an open reading frame starting with the initiation codon ATG and terminating with the triplet TAG within the acquired cellular sequences and terminating with the triplet TAG at a point thirty-three nucleotides into helper viral sequences to the right of the helper-viral-cellular junction. The stretch of 795 nucleotides would code for a protein of 265 amino acids with a molecular weight of 30,000 daltons. The eleven amino acids at the carboxy terminus of such a protein would be derived from the *env* gene of helper virus. Antibodies were prepared against synthetic peptides derived from the predicted amino acid sequences. One such antibody precipitated two magnesium proteins of apparent nucleotide weight of 30,000 daltons and 51,000 daltons.

B. Introduction

The mechanism by which acute transforming retroviruses have acquired their ability to transform cells is closely associated with their capacity for synthesis of double-stranded DNA copies of their RNA genome. The DNA proviral forms cannot only integrate into host chromosomes, but have the ability to acquire host genetic material by a process of recombination. The replacement of viral genes by cellular ones usually results in the virus becoming defective, requiring the presence of non-defective helper virus for the maintenance of the acquired genes [1]. On rare occasions, the captured sequence contains a crucial control gene which, when inserted by the virus into cells which do not normally express it, or into sites in the cell where its expression is not regulated, can result in malignant transformation of the cells. In this report we present the nucleotide sequence of the transforming gene of AMV and identification of its translational product. This unique virus causes acute myeloblastic leukemia with a short latent period in chickens [2]. In vitro, AMV transforms a specific class of hematopoietic cells, but does not morphologically transform fibroblasts [3, 4]. Only certain target cells are responsive to the AMV *onc* gene product which induces proliferation of immature and altered hematopoietic cells, i.e., leukemic myeloblasts.

C. Results

I. Restriction Enzyme Map and Strategy of Sequencing the Transforming Gene of AMV

The upper portion of Fig. 1 shows the genetic map and orientation of the AMV provirus in the avian chromosome. An important structural feature of the integrated AMV genome is the occurrence of two large terminal repeats (LTRs) of 285 bases each at both the 5′ and 3′ ends of the pro-

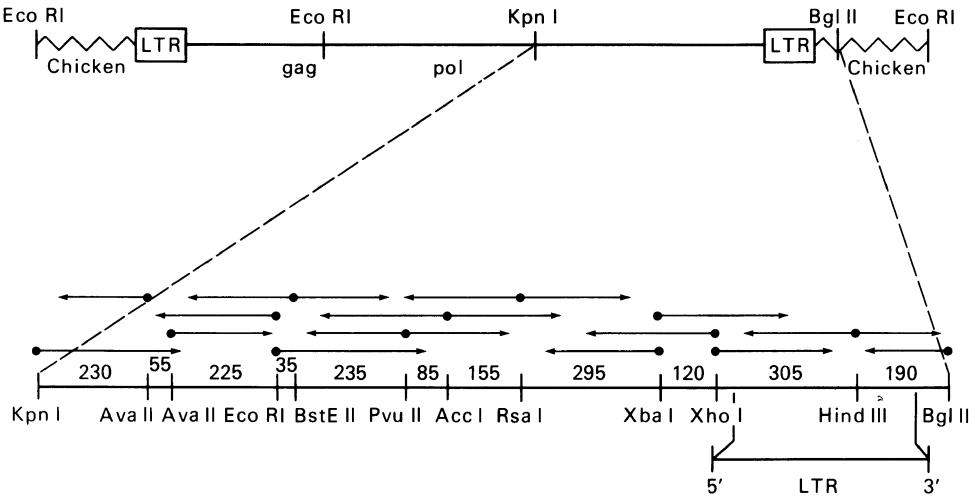

Fig. 1. Restriction enzyme map and strategy of sequencing the transforming gene of AMV. The genome is sequenced using the restriction sites indicated as the diagrammatic map. The 5′ ends were labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled end of each fragment is indicated by the *filled circle* and the extent and direction of sequencing are indicated by *arrows*

viral sequence [6]. Within the proviral sequences, we can identify the *gag* gene proximal to the 5′ end of the viral RNA, followed by the polymerase gene and the oncogenic sequences. The oncogenic sequences extend to the 3′ end of the viral RNA and beyond the *Kpn* site of the cloned provirus (Fig. 1).

The lower portion of Fig. 1 provides a summary of the strategy employed to determine the nucleotide sequence. After digestion of DNA with appropriate restriction enzymes, the fragments to be sequenced were isolated on agarose gels or polyacrylamide gels and sequenced in either the 5′→3′ or 3′→5′ direction. The fragment is oriented from left to right, 5′ to 3′, with respect to the viral RNA. The *arrows* below the DNA strand indicate the length and direction of sequencing.

II. Nucleotide Sequence of the Transforming Gene of AMV

The nucleotide sequence of the 3′ end of the integrated AMV provirus is shown in Fig. 2. Within this sequence we can identify several domains: (1) the terminal portion of the polymerase gene, identified by an open reading frame extending from position 1 and terminating with a TAG codon at position 162; (2) a region of 350 bases without on apparent open reading frame extending between positions 165–515; (3) an open reading frame of 795 bases extending from positions 516–1310; and (4) the 3′-LTR adjacent to the host sequences.

Earlier studies have revealed that the AMV genome has undergone recombination in which the entire helper virus *env* gene has been replaced by cellular sequences [7, 8]. In order to localize the points of recombination, we have compared the carboxy terminal sequence of the AMV polymerase gene with that of the nondefective Prague strain of Rous sarcoma virus (PR-RSV) (Schwartz D, personal communication). From position 1 to position 78 of the AMV DNA fragment sequenced here, the nucleotide sequence is identical to that of PR-RSV. From position 78 to the termination signal, TAG at position 1313, the sequences of AMV and PR-RSV are entirely different, thus localizing the 5′ end of the cellular insertion sequences.

It is interesting to note that the host-helper virus junction occurs at a region which constitutes a potential splice acceptor site. In general, splicing acceptor sites (at the 3′ end of the intervening sequence) contain a pyrimidine-rich nucleo-

tide tract followed by the sequence AGG. The junction point between the cellular insertion sequence and the helper viral sequence fits the consensus acceptor splice sequence [9].

The product of the AMV transforming gene has yet to be identified. Examination of the cellular-derived *amv* sequences (Fig. 2) reveals an open reading frame starting with the initiation codon ATG at position 516 and terminating with the triplet TAG at position 1310. This stretch of 795 nucleotides could code for a protein of 265 amino acids with a molecular weight of 30,000. The amino acid sequence predicted from this region is also shown in Fig. 2.

Ribonuclease T_1-resistant oligonucleotide analysis was carried out by Duesberg et. al [8] on RNA isolated from the defective AMV particles. The RNA from these particles contains 14 unique T_1-oligonucleotides which are unrelated to sequences present in nondefective avian retroviruses and to the transformation-specific sequences of other avian leukemia and sarcoma viruses. Duesberg et al. suggested that these RNA sequences belong to the leukemogenic region of the AMV geneome [8]. We have utilized the computer program devised by Queen [10] and have positively identified 13 of the 14 oligonucleotides in the sequence presented in Fig. 2. Table 1

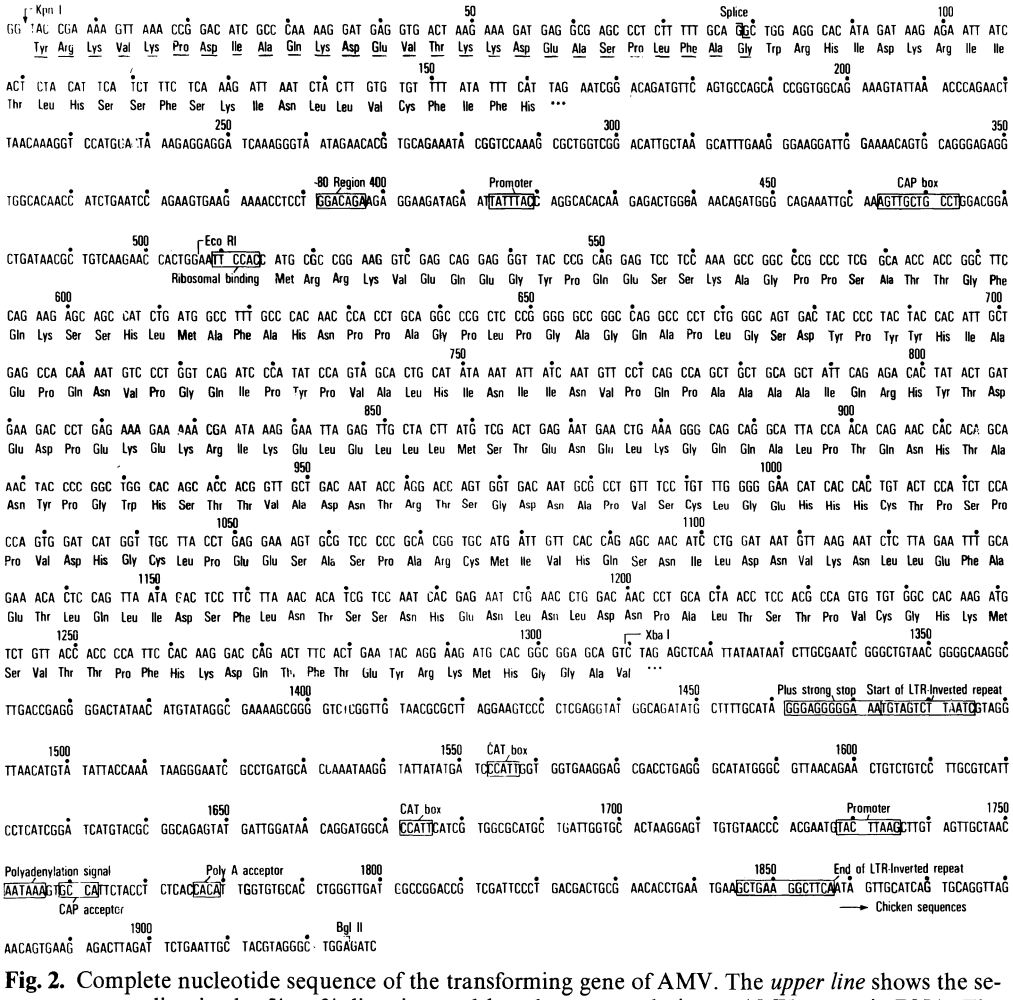

Fig. 2. Complete nucleotide sequence of the transforming gene of AMV. The *upper line* shows the sequence proceeding in the 5′ to 3′ direction and has the same polarity as AMV genomic RNA. The amino acid sequence deduced from the open reading frame is given in the *bottom line.* The major structural features of the genome are indicated

Table 1. The AMV-specific T_1 oligonucleotides and their position in the sequence: 101 (etc.) indicates the number for the nucleotide chromatographic patterns in [10]

T_1 Oligo	Sequence	Position in the sequence	Specificity
101	ATTAATCTACTTG	132 – 144	AMV specific
102	AATTATCACTCTACATTCATCTTTCTCAAAG	101 – 131	AMV specific
103	CACTAACCTCCACG	1207 – 1220	AMV specific
104	AATTATTTACCAG	410 – 422	AMV specific
105	TTTTATATTTCATTAG	149 – 164	AMV specific
106	ACTACCCCTACTACCACATTG	679 – 699	AMV specific
107	CCCACAACCCACCTG	622 – 636	AMV specific
108	CATATAAATATTATCAATG	747 – 766	AMV specific
113	CATTACCAACACAG	892 – 905	AMV specific
110	CAAACTACCCCG	916 – 927	AMV specific
111	ACTCCTTCTTAAACATCG	1153 – 1172	AMV specific
112	TACTCCATCTCCACCAG	1013 – 1029	AMV specific
114	TTACCACCCCATTCCACAAG	1246 – 1265	AMV specific
51	CTCAATTATAATAATCTTG	1316 – 1334	Common C-region
52	TATATTACCAAATAAG	1499 – 1514	LTR (U_3)
53	CACCAAATAAG	1529 – 1539	LTR (U_3)
54	CTAACAATAAAG	1746 – 1757	LTR (U_3)
55	?	?	
56	TCATTCCTCATCG	1616 – 1628	LTR (U_3)
57	CACCATTCATCG	1669 – 1688	LTR (U_3)
1	CCATTCTACCTCTCACCACATTG	1760 – 1782	LTR (U_3)

lists the T_1-oligonucleotides identified by Duesberg [8] and their position within our sequence. Comparison of our sequence with that of the RSV envelope region (Schwartz D, personal communication) reveals that the last 11 amino acids at the carboxy terminus are shared by the two proteins, suggesting that the *amv* gene is incomplete and utilizes the envelope terminator codon. This positions the 3′ terminus of the recombination event at position 1277.

III. Identification of the Transforming Gene (*amv*) Translational Product

Nucleotide sequence analysis of *amv* has revealted the presence of a 795-base open reading frame commencing within the acquired cellular sequences and terminating within the helper viral sequences (Fig. 2). Synthetic peptides prepared on the basis of predicted amino acid sequences of various genes have been utilized in the recent past to prepare antibodies against such proteins. Such antibodies provide a powerful tool for identificaiton and characterization of proteins that could not be previously identified. If the *amv* reading frame were functional, antibodies prepared against the synthetic peptides predicted from this sequence should be capable of precipitating the translational product of this viral *onc* gene. Such an approach not only allows the detection of transforming proteins but also provides additional experimental evidence for the correctness of the open reading frame derived by nucleotide sequence analysis. For this, we chemically synthesized three peptides, each 15 amino acids long. The peptides were coupled to thyrogloblin using 2-ethyl carbondiinide [21] and used for immunization of rabbits.

In an attempt to identify the *amv* translational product, the myeloblasts nonproductively infected with AMV were labeled for 6 h with [^{35}S]-methionine and the cell lysates prepared by detergent lysis. The cytoplasmic extracts were then immunoprecipitated with preimmune sera or anti *amv*-1, anti *amv*-2, and anti *amv*-3. As

negative controls, we used uninfected cells derived from chicken embryo fibroblasts. As shown in Fig. 3, two proteins with an apparent molecular weight of 30,000 daltons and 57,000 daltons were precipitated with anti *amv*-2. These proteins were not precipitable with preimmune sera nor were they detectable by any of the sera used in control cells that were not infected by AMV. Also, these two proteins were not precipitable from cells infected with helper virus alone. These observations strongly suggest that p30 and p57 are encoded by the transforming region of *amv*.

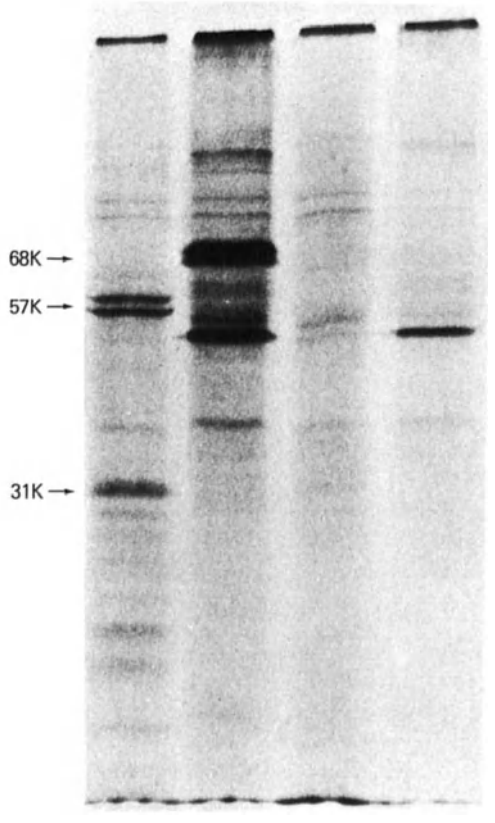

Fig. 3. SDS-PAGE analysis of immunoprecipitated cell lysates. Panel numbering is from *left* to *right*. *Panel 1,* AMV-transformed nonproducer cells + anti *amv*-2. *Panel 2,* chicken embryo fibroblasts + anti *amv*-2. *Panel 3,* AMV-transformed nonproducer cells + prebleed of anti *amv*-2. *Panel 4,* chicken embryo firbroblasts + prebleed anti-*amv*-2. Pr68 in *panels 2 and 4* has been identified to be actin

D. Discussion

A message generated from the AMV-transforming region should direct the synthesis of the transforming protein with the predicted amino acid sequence shown in Fig. 2. This messenger RNA could be generated either by splicing with the leader sequence derived from the 5′ terminus of genomic RNA or by splicing with the leader sequence derived from the 5′ terminus of genomic RNA or by independent promotion.

There are at least four transcription and translation regulatory sequences representing a minimum of 31 properly arranged nucleotides within a region 124 nucleotides immediately upstream from the putative leukogenic sequence. The arrangement of these nucleotides cannot be due to chance and indicates that the *amv* insert was probably not acquired by recombination between viral DNA and a cDNA copy of cellular mRNA transcribed from the *c-amv* sequences. The creation of the AMV genome may be explained by a deletion-recombination mechanism first postulated for the formation of the Abelson virus genome by David Baltimore (personal communication). According to the model, a MAV provirus with a large deletion in the 3′ half of the viral genome starting at position 78 beyond the *Kpn*I site (Fig. 2) would have been integrated in the vicinity of the chicken *amv* sequences. Transcriptions initiated by the MAV 5′-LTR generated a hybrid MAV-chicken RNA extending from the remainder of the viral genome to the 3′ terminus of the cellular c-*amv* sequences. Subsequent splicing within the *c-amv* sequences which contain three introns not present within AMV then generated a hybrid viral chicken mRNA terminating at the *myb* 3′ terminus. This hybrid mRNA could have been packaged in a MAV virion and subsequently copied in cDNA by the viral reverse transcriptase. This was followed by recombination with MAV DNA to create AMV by addition of the MAV 3′ end sequences starting at position 1316 beyond the *Kpn*I site. Splicing is generally used in the synthesis of viral subgenomic message. Leader sequences identified in MC29 [11] and RSV (Schwartz D, personal communication) cloned proviruses contain the 5′-

LRT, a noncoding region and 18 nucleotides coding for six amino acids of the N-terminal portion of the viral protein p19 ([11]; Schwarz D, personal communication). The splice donor portion of these sequences agrees with the consensus splice sequence of eukaryotic genes [9].

The alternate model for controlling the expression of the transforming gene would utilize the transcriptional signals found within the cellular insertion sequences in the region which lies between the polymerase gene and the open reading frame (Fig. 2). This type of independent promotion would not utilize the transcription controls of the viral 5′-LTR. Within the 350 base pair region in front of the putative leukemogenic sequence we have identified transcriptional signals similar to those present in other eukaryotic genes [12–20]. A six-base AT-rich sequence characteristic of eukaryotic promotors was identified at position 413–417, −56 bp from the capping site. Similarly, signals such a −80 bp region

Table 2. Landmarks of AMV as suggested by DNA sequences

−80 region				
Sequence	Source	Homology	Distance from $\overset{-1}{A}C\rightarrow$	Reference
GGACAGA	AMV	6/7	−79	
GGACAAA	Conalbumin		−78	[12]
Promoter region				
Sequence	Source	Homology	Distance from $\overset{-1}{A}C\rightarrow$	Reference
TATAAAT	General		−20 to −30	[13]
TATTTAC	AMV	4/7	−56	
TATTTAT	Ad 2 early	5/7	−23	[14]
TATATAT	Ovalbumin	6/7	−24	[15]
"CAP" box (AC→)				
Sequence	Source	Homology	Distance from ATG	Reference
GTTGCTCCT . . . . . $\overset{+1}{A}C$	General		Variable	[16]
AGTTGCTGCCT . . $\overset{+1}{A}C$	AMV	9/10	−39	
AGTTGCT · CCT . . $\overset{+1}{A}C$	β-globin^{maj}	10/11	−43	[17]
Initiator ATG region				
Sequence	Source	Homology		Reference
C/AAAPy*ATG*	General			[18]
C AC C*ATG*	AMV	7/7		
C AA C*ATG*	Conalbumin	6/7		[12]
A AC C*ATG*	Mouse α-globin	6/7		[19]
Ribosome binding				
Sequence	Source	Homology	Distance from ATG	Reference
TTCCGC	General		Variable	[20]
TTCCAC	AMV	5/6	−7	

and ribosomal binding sites have also been identified within this region. If these signals were to be utilized for the transcription of the *v-amv* gene, this would be the first example of a case in which the virus has incorporated the cellular regulatory signals for the transcription of its *onc* gene.

It would be very difficult at this point to conclude that these proteins are different or related to each other. It is possible that the p57 is a modified version of p30 (glycosylation, phosphorylation, etc.). Alternatively, it is possible that the two different proteins are generated from the same reading frame by two different mechanisms. The p30 protein could be the translational product of a mRNA derived by independent promotional signals (Table 2) identified in the transforming region. The p57 protein could be derived from a spliced mRNA generated from leader sequences provided by the helper virus and spliced to a region of the *amv* sequences 438 bases upstream from the ATG of the open reading frame. If this latter possiblility exists, a suppressor tRNA should be available in order to suppress translational terminator signal (TAA) at position 308 (Fig. 2). Alternatively, the p57 could be a translational product of *c-amv* encoded mRNA which would be expected to be much larger at the carboxy terminus.

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DNA-Binding Ability of Transforming Proteins from Avian Erythroblastosis Virus and Mutant Avian Myelocytomatosis Virus, MC29, in Comparison with MC29 Wild Type

K. Moelling, I. Greiser-Wilke, M. K. Owada, P. Donner, and T. Bunte

A. Introduction

Oncogenic RNA tumor viruses code for oncogenes, which induce tumors in the animal and malignant transformation of cells in culture. The oncogenes are derived from normal cellular genes and their expression is controlled by viral regulatory elements which results in a roughly 100-fold amplification of the gene in comparison to its normal expression [9]. Since several viral oncogenes are expressed as hybrid proteins consisting of viral structural elements fused to transformation-specific regions [7], analysis of transforming proteins can be experimentally approached by antibodies against the viral structural part which allow the identification of the transforming proteins. We have analyzed such proteins from representatives of different classes of acute leukemia viruses, the acute avian myelocytomatosis viruses, MC29, and the acute avian erythroblastosis viruses, AEV, both of which affect the hematopoietic system of the infected animal at various stages of differentiation and cause rapid death [5]. Furthermore, they transform fibroblasts in culture. The transformation-specific protein from the third class of viruses, from avian myeloblastosis viruses, AMV, cannot be analyzed by this approach, as it is not expressed as *gag-onc* fusion protein [9]. We have recently identified the MC29 transforming protein *v-myc*, a molecule of 110K, as a DNA-binding protein [3]. Here we demonstrate that the *v-myc* protein, if purified from a transformation-defective MC29 deletion mutant, Q10C, which has a molecular weight of 95K instead of 110K and has reduced transformation ability [11], no longer binds to DNA. Furthermore, the purified AEV-specific protein, a molecule of 75K [8], also does not bind to DNA. Nothing is so far known about its function. Our results indicate that avian oncornaviruses carry at least three different types of oncogenes which cause transformation in vivo and in vitro, probably by different mechanisms. One of these mechanisms involves a DNA-binding protein, and a second one a protein kinase, while nothing is known about the third mechanism.

B. Methods

All methods have been described by Donner et al. [3] and Moelling et al. [10].

C. Results

Fluorescence microscopy of MC29-Q8-NP cells, which is a MC29-transformed quail fibroblast cell line [1], and Q10C cells, from a quail fibroblast cell line transformed by a deletion mutant of MC29 [11], was performed with monoclonal antibodies against the structural protein p19 [6], which represents the N-terminal portion of the 110K and 95K *v-myc* proteins. The procedure for indirect immunofluorescence with fluorescein-labeled second antibodies has been described previously [3]. The cells (Fig. 1) show strong nuclear fluorescence in both cases. In contrast, AEV-transformed fibroblasts exhibit cytoplasmic fluorescence.

The transformation-specific proteins from ^{35}S-methionine-labeled MC29-Q8-

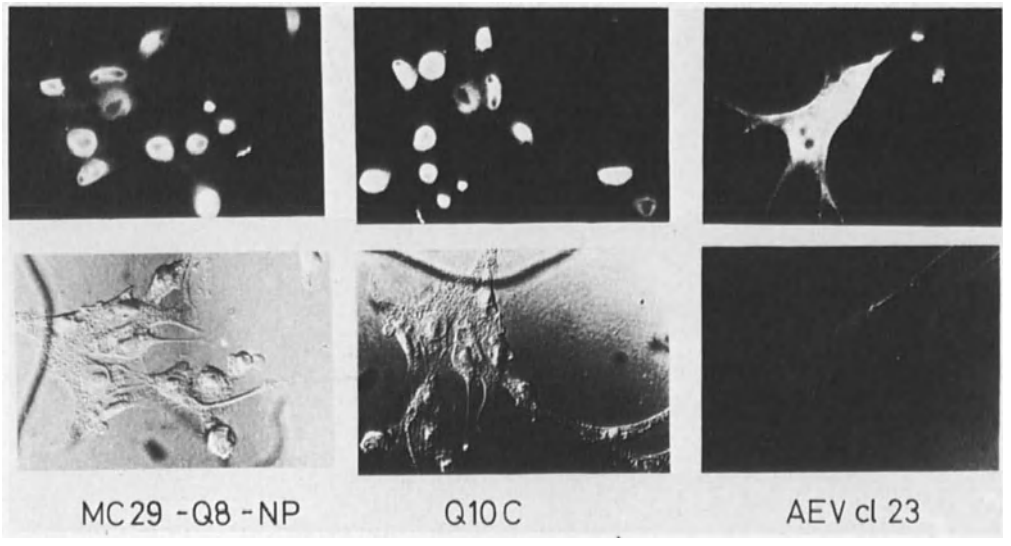

Fig. 1. Immunofluorescence of MC29-transformed quail fibroblasts (MC29-Q8-NP), of MC29 deletion mutant-infected quail fibroblasts Q10C, and of AEV-transformed chicken fibroblasts. All cells were nonproducers. Indirect immunofluorescence was performed using monoclonal antibodies against p19 [6], the N-terminus of the transforming proteins, and FITC-labeled anti-mouse IgG (Miles-Yeda, Miles Labs, Elkhart, Indiana/USA). The method has already been described [3]. *Bottom pictures:* Nomarski interference contrast of the same field. ×515

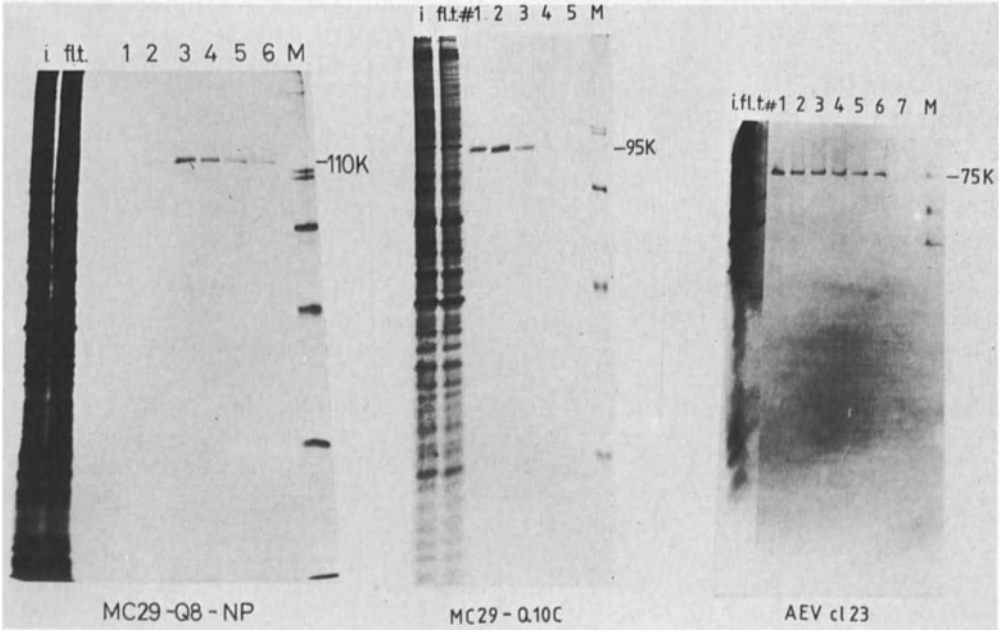

Fig. 2. Purification of 110K proteins from MC29-Q8-NP, 95K proteins from Q10C, and 75K proteins from AEV cl 23 using immunoaffinity column chromatography with monoclonal IgG against p19. Details have been described [3, 6]. Cells were labeled with ^{35}S-methionine for 2 h and lysed. Aliquots of the input (5 µl out of 15 ml), flow-through (5 µl out of 15 ml), and eluted material (25 µl out of 1 ml) were applied to a 10% polyacrylamide gel which was exposed for autoradiography. *M* indicates marker proteins

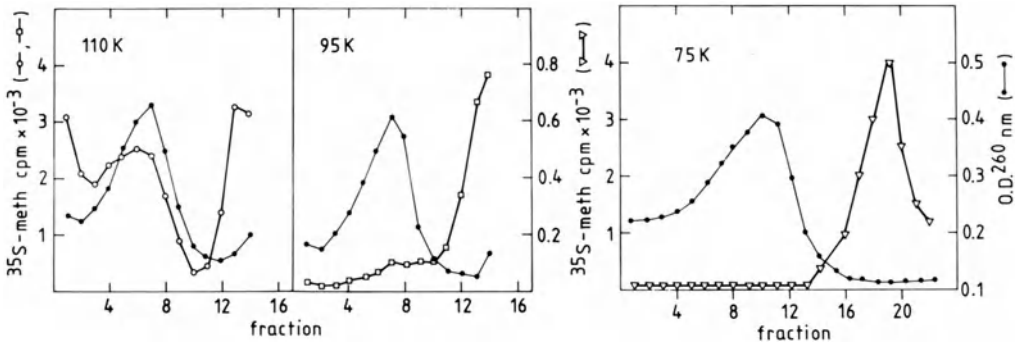

Fig. 3. DNA-binding of purified transforming proteins. ^{3}H-labeled chicken DNA sheared to about 10 kb was mixed with the ^{35}S-methionine-labeled purified proteins in the presence of 50 m*M* NaCl, sedimented through a 10%–30% glycerol gradient in 50 m*M* Tris-HCl, pH 8, and fractionated. The radioactivity of the fractions was determined by liquid scintillation counting

NP, Q10C, and AEV cl 23 cells were purified by immunoaffinity column chromatography with monoclonal antibodies against p19. The proteins were eluted with a low pH buffer and aliquots from the eluted fractions were analyzed by polyacrylamide gel electrophoresis. The result of the purification is shown in Fig. 2. The input and flow-through material is shown for comparison. The purification is about 3000-fold.

The purified proteins were then analyzed for their ability to bind to DNA. ^{3}H-labeled double-stranded DNA was sheared to about 10 kb in size, mixed with the purified proteins, and analyzed by sedimentation analysis. Only the wild-type *v-myc* protein cosedimented with the DNA by binding to it (Fig. 3), while the protein remained on top of the gradient in the absence of DNA (not shown). The deleted 95K protein from the Q10C mutant as well as the 75K protein from AEV cl 23 did not bind to DNA (Fig. 3).

D. Discussion

As recently shown, the transformation-specific protein *v-myc* from MC29 virus is localized in the nucleus and binds to double-stranded DNA [3]. Our results indicate that the DNA-binding correlates with transformation at least of bone marrow cells, as the protein from Q10C cells transformed by the MC29 deletion mutant lost its DNA-binding property together with part of its transformation capacity. While the MC29 deletion mutants have a markedly reduced transformation potential for bone marrow cells in vivo and in vitro, the transformation ability of fibroblasts in vitro remains unaffected [11]. It can be concluded that apparently three unrelated mechanisms can result in malignant transformation of fibroblasts by oncornaviruses. The first mechanism involves the plasma membrane-associated protein kinase from avian sarcoma viruses [4], and the second mechanism a nuclear protein which binds to DNA. More details of the protein-DNA interaction need to be elucidated to understand this last mechanism. Little is known about a third mechanism, transformation by AEV, which involves two specific proteins. One of these, p75$^{gag\text{-}erbA}$, is not a protein kinase, is not a nuclear protein [10], and does not bind to DNA. Nothing is known about the localization or function of the second protein, *erbB* [2], which may also be involved in the transformation process.

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Lack of Expression of Cellular Homologues of Retroviral *onc* Genes in Bovine Tumors*

R. Kettmann, E. H. Westin, G. Marbaix, J. Deschamps, F. Wong-Staal, R. C. Gallo, and A. Burny

A. Introduction

Bovine leukemia virus (BLV), an exogenous retrovirus of cattle [5, 11], induces B-lymphocyte neoplasms (termed enzootic bovine leukosis, EBL) after long latent periods [2]. BLV contains no host cellular sequences and does not appear so far to bear genes capable of inducing transformation directly. A wide range of genomic sites can accomodate BLV proviruses. Transcription of viral DNA including long terminal repeated sequences has not been detected, strongly suggesting that viral gene expression is not required for maintenance of the tumor state. No expression of 3′-proximate cellular sequences has been observed, indicating that proximate downstream promotion did not take place in the cases examined [12].

The transforming genes of retroviruses are derived from normal cellular genes (*c-onc*) conserved among vertebrates [6, 17]. There is good evidence that virus-induced transformation is correlated with enhanced levels of expression of these genes [3, 14, 15]. Using labeled molecularly cloned DNA probes containing viral *onc* sequences, expression of cellular homologues of retroviral *onc* genes has been found in human tumor cells [7, 19]. Using the same approach, we examined whether or not one of these known *onc* genes was expressed at high level during the maintenance of the tumor state in bovine lymphosarcoma.

B. Results and Discussion

The viral *onc* genes used in the present investigation are listed in Table 1. Nick-translated DNA probes of each viral *onc* gene were first analyzed for their ability to detect homologous sequences in bovine DNA. Normal bovine cellular DNA was cleaved with *Eco*RI. The DNA fragments were subjected to agarose gel electrophoresis and to Southern blotting analysis in relaxed hybridization conditions [19]. As shown in Fig. 1 (lane 1), the *abl* DNA probe detected five DNA fragments of 6.2, 3.4, 2.8, 1.8, and 1.2 kb. The signal intensity was highest with the *abl* DNA probe, probably reflecting its greater homology with bovine DNA as compared to that of other viral *onc* genes tested. The other *onc* DNA probes (myc, erb, myb, src, ras, fes, and sis; Table 1) detected one or at most a few DNA fragments (data not shown). DNA from EBL tumors were analyzed in parallel (Fig. 1, lanes 2–7). For each *onc* probe tested, the patterns obtained were identical to the one observed with normal bovine DNA. These results indicate that in none of the EBL DNAs was there obvious rearrangement of any *onc* genes due to, for example, integration of the BLV provirus. The approach used to detect *onc*-related transcripts in bovine tumors involved isolation of poly (A)-containing RNAs and analysis by dot blot hybridization on nitrocellulose filters in relaxed hybridization

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Table 1. The different viral *onc* probes

Species	Virus strain	*onc* sequence	Viral clone obtained from
Avian	Avian myeloblastosis (MC 29)	myc	Dr. T. Papas [13]
	Avian erythroblastosis (AEV)	erb	Dr. M. Bishop [18]
	Avian myeloblastosis (AMV)	myb	Dr. M. Baluda [1]
	Rous sarcoma (RSV)	src	Dr. M. Bishop [4]
Murine	Abelson murine leukemia (Ab-MuLV)	abl	Dr. S. Aaronson [16]
	Harvey murine leukemia (Ha-MuSV)	ras	Dr. M. Martin [10]
Feline	Snyder-Theilen feline sarcoma (ST-FeSV)	fes	Dr. C. Scherr [8]
Simian	Simian sarcoma (SSV)	sis	Dr. R.C. Gallo [9]

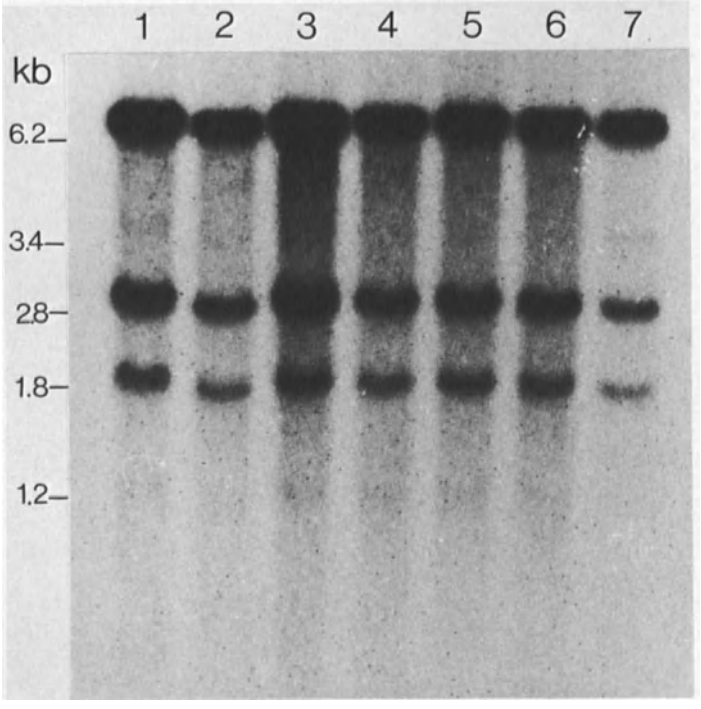

Fig. 1. Detection of bovine DNA sequences related to the *abl* retroviral *onc* gene. High-molecular weight DNA (10 μg) from normal bovine leukocytes (*lane 1*) and from EBL tumors (*lanes 2–7*) was digested with *Eco*RI and electrophoresed on an 0.8% agarose gel. Southern blot was prepared and incubated for 24 h with 2×10^6 c.p.m./ml nick-translated (2×10^8 c.p.m./μg) viral *onc* probe prepared from cloned DNA from Abelson MuLV. Hybridization was performed for 16 h at 37 °C in 50% formamide and 3×SSC with 10% dextran sulfate and followed by washings at 60°C with 2×SSC. The blot was exposed for 1 week using Kodak XAR-5 film and Dupont Cronex Lightning Plus screens. *Eco*RI generated fragments of bacteriophage λ DNA were used as molecular weight standards. *kb*, kilobase pairs

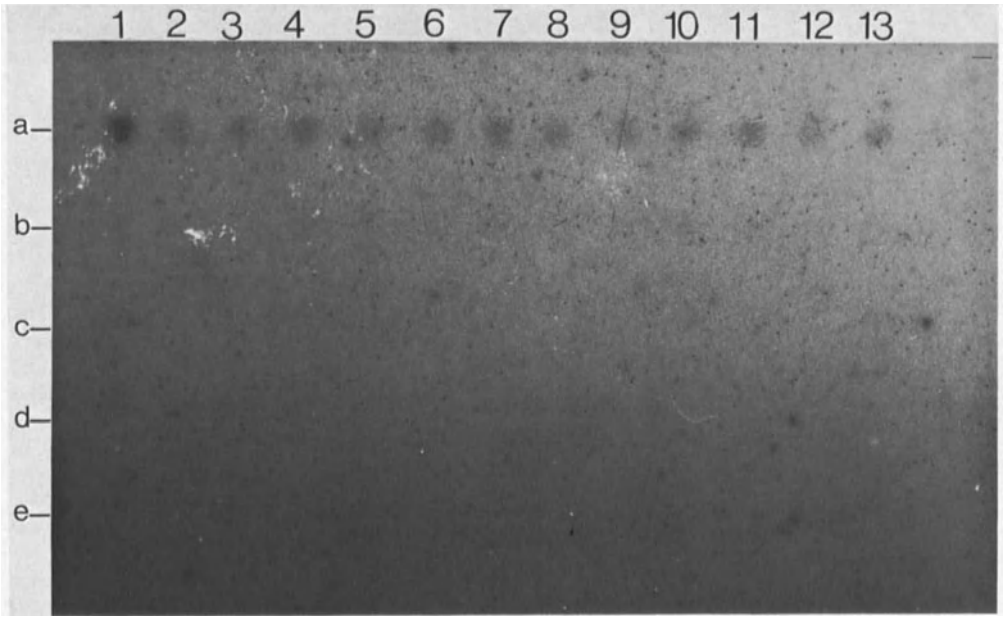

Fig. 2. Dot blot assay of poly (A)-selected RNA from leukocytes of a normal animal (*lane 1*) and from tumors of several other animals (*lanes 2–13*). Fivefold dilutions were tested, from 2 μg (*a*) to 3.2 ng (*e*) of poly (A)-selected RNA. The dot blot preparation was hybridized with 2×10^6 c.p.m./ml nick-translated (2×10^8 c.p.m./μg) cloned DNA from Abelson MuLV. Hybridization was performed in 50% formamide, 5×SSC with 10% dextran sulfate at 37 °C for 16 h. Filters were washed four times in 2×SSC/0.1% SDS at room temperature for 5 min each and then in 1×SSC/0.1% SDS at 42 °C for 15 min each. Autoradiography was a 1-week exposure

conditions. This technique allowed us to detect as little as 1 pg of complementary RNA. Bovine tumor cells were tested for the presence of viral *onc*-related transcripts both as total and poly (A)-selected RNAs. Figure 2 shows the hybridization between ^{32}P *abl* DNA as a probe and poly (A)-selected RNAs from normal leukocytes (lane 1) and EBL tumors (lanes 2–13). No quantitative difference between the signals observed for the RNA from normal tissue and the RNAs from EBL tumors was observed. The same conclusion held true for the other *onc* DNA probes tested. Thus it appears that none of the *onc* genes tested were implicated in the maintenance of EBL tumors by a mechanism involving enhanced expression of these genes.

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Molecular Dissection of the Bovine Leukemia Virus Envelope Glycoprotein (BLV gp51) by a Monoclonal Antibody Study*

C. Bruck, D. Portetelle, J. Zavada, and A. Burny

A. Introduction

Neoplasms of the lymphatic tissue in the bovine species can be classified into two major types according to clinical and epidemiological data:
1. The juvenile form of bovine leukosis, with three different clinical forms: the multicentric, thymic, and cutaneous forms. The juvenile form is rare and shows a random geographical distribution. It has also been called sporadic bovine leukosis.
2. The adult form of bovine leukosis leads to more diversely located lymphosarcomas, mostly of the B-lymphocyte lineage and/or to B-cell leukemias. The adult form of bovine leukosis is more common, but restricted to geographically limited regions. Although it affects only a small percentage of the cattle population, it behaves as a typical herd disease. Its distribution pattern is typically one of transmissible diseases and has allowed the classification of the adult form of lymphosarcomas as "enzootic bovine leukosis" [1].

In 1969 Miller et al. identified viral particles in short-term cultures of leukocytes of animals in persistent lymphocytosis [2]. Transmission experiments [3] and seroepidemiological studies [4] finally established that this virus is an etiological agent of enzootic bovine leukosis. This virus, called bovine leukemia virus (BLV), can be experimentally transmitted to sheep, goats, primates, rabbits, and other mammals by intravenous, intradermic, or oral administration of infected lymphocytes. Sheep are highly susceptible to leukosis induction by BLV, goats rarely develop BLV lymphosarcomas, and other animals, although persistently infected by BLV, seem to be resistant to the oncogenic properties of this virus [1]. Only one case of tumor induction in goats by BLV has been reported so far [5]. In natural conditions, BLV is transmitted between cattle by "close contact". The exact mode of transmission in the field condition is still unclear: transmission by infected animals, secretions and milk, blood-sucking insects, and nonsterile veterinary instruments have been reported.

Molecular analysis of BLV has made possible its classification as a retrovirus (60–70S genomic RNA, reverse transcriptase) [6]. Hybridization experiments with cDNA complementary to genomic viral RNA have established the following points:
1. BLV is an entirely exogenous virus: integrated proviral BLV DNA is only detectable in infected B-lymphocytes [7].
2. No proviral DNA can be found in sporadic leukosis tumors [8]; enzootic leukosis tumors always contain one or several copies of proviral DNA. The integration site of the BLV genome is variable from case to case [9].
3. The three viral genes necessary for the virus life cycle have been identified on the BLV genome (*gag, pol, env*), showing that BLV is a nondefective leukemia virus [10].

Immunochemical studies have led to the identification of the viral glycoproteins and proteins [1]: gp51 and gp30 are envelope

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antigens; p24 is the major core protein; reverse transcriptase is associated with the genomic RNA; p15, p12, and p10 are internal proteins with so far undefined location and function.

BLV is genetically and antigenically unrelated to other animal retroviruses and was considered as a group of its own until the discovery of a human T-cell lymphoma retrovirus HTLV in 1981 [11]. The major internal core protein of HTLV p24 shares amino acid sequence homology with BLV p24, and HTLV and BLV p12 display common antigenic determinants [12]. Like HTLV, BLV is a weakly oncogenic virus: there is a long latency between the moment of BLV infection and the onset of the tumor phase, and only a small percentage of the infected animals die with leukemia: in Japan 30%–50% of clinically normal cattle in an endemic area became infected with BLV, while the incidence of lymphosarcoma or leukemia in this area was less than 0.1% [13].

In about 30% of infected cattle, BLV infection leads to a nonmalignant hematological disorder characterized by an elevated but stable lymphocyte count, called persistent lymphocytosis (PL). As shown by molecular hybridization, the expanded lymphocyte population in the PL phase is of polyclonal origin: the proviral BLV is integrated at many different sites, whereas the tumor phase is monoclonal [14]. These observations, together with the weak oncogenic activity of BLV, suggest that a condition superimposed on BLV integration is necessary to induce neoplastic transformation of lymphoid cells.

Natural or experimental infection of cattle with BLV induces a vigorous antibody response, which is often the only constant feature of BLV infection during the latency period of the disease. Antibodies are directed mainly against the envelope glycoprotein gp51 and the internal core protein p24 [1]. Antibodies to gp51 are consistently formed at a higher titer than antibodies to p24 and can be detected earlier after experimental infection [15]. In spite of their high titer, antiviral antibodies do not seem to be protective against the onset of the leukemic phase: although subject to major fluctuations, the antibody titer rises constantly during the progression of the disease and reaches maximal level at the death of the animal in the tumor phase [16].

May be as a consequence of antigenic modulation, BLV-infected lymphocytes do not express viral antigens in vivo [17]. This could explain why antiviral antibodies produced in response to BLV infection are unable to inhibit the outgrowth of leukemic clones. However, passive immunization of calves with colostral antibodies is protective against primary BLV infection [18], suggesting that efficient vaccination against primary BLV infection should be possible.

Previous studies have suggested that the natural anti-gp51 antibody response is focused on a single antigenic region of gp51. Exoglycosidase treatment of gp51 abolishes the reactivity of these antibodies against the antigen, suggesting that the structure of the relevant antigenic region is directly or indirectly determined by the carbohydrate moiety of the molecule [19]. These natural anti-gp51 antibodies produced by infected cattle display several antiviral activities: they neutralize virus infectivity [20] and syncytia-inducing activity [21] and exhibit a strong cytolytic effect on BLV-producing cells in the presence of rabbit complement [16]. In order to characterize the regions of gp51 which are indispensable for an efficient BLV vaccine, monoclonal anti-gp51 antibodies were produced.

B. Results

Fifteen monoclonal anti-gp51 antibody-secreting hybridoma cell lines were obtained by fusing spleen cells of a Balb/c mouse immunized with partially purified gp51 with a subclone of the SP_2 myeloma cell line. These antibodies were classified into high- and low-avidity antibodies according to the slope and plateau values of their titration curves obtained by ELISA titers on polyethylene immobilized gp51 (see Table 1). Competition experiments between these different antibodies registered the fixation of radiolabeled antibodies in the presence of an excess of cold competition antibody. This test allowed the identification of eight nonoverlapping gp51 epitopes (A–H). Partially overlapping sites have been called B and B′, and D and D′ (see Table 1, Fig. 3).

Table 1. Pseudotypes and early polykaryocytosis: inhibition activities of 15 Anti BLV gp51 Monoclonal antibodies

Antibody number	Ig isotype	Binding activity on plastic-adsorbed gp51	Site specificity	Number of plaques in PI test	Number of Syncytia in EPI
GA1	IgG1	High	A	155	233
GA2	IgG1	High	B	102	268
GA3	IgG1	Low	H	50	1
GA4	IgG1	Low	F	39	151
GA5	IgG1	High	D	140	210
GA6	IgG2b	Low	E	102	258
GA7	IgG1	Low	E	120	210
GB8	IgG1	High	C	100	298
GB9	IgG1	High	A	121	225
GB10	IgG1	High	B′	114	210
GB11	IgG1	High	A	125	320
GA12	IgG1	Low	E	NT	NT
GA13	IgG1	Low	H	4	0
BC14	IgG2a	Low	G	33	125
GA15	ND	High	D′	127	265

In order to localize these antigenic sites on the gp51 molecule, we performed limited protease (urokinase) digestion of the antigen, followed by radioimmunoprecipitation (RIP) with monoclonal antibodies and SDS-PAGE analysis. The results show that the eight sites are distributed on two distinct fragments of gp51, sites A–D on fragment I, mol. wt. 35,000, sites E–H on fragment II, mol. wt. 15,000 (antibodies against sites E–H recognize each of the four bands of different molecular weight, suggesting that urokinase digestion of gp51 is incomplete) (see Table 1, Fig. 1).

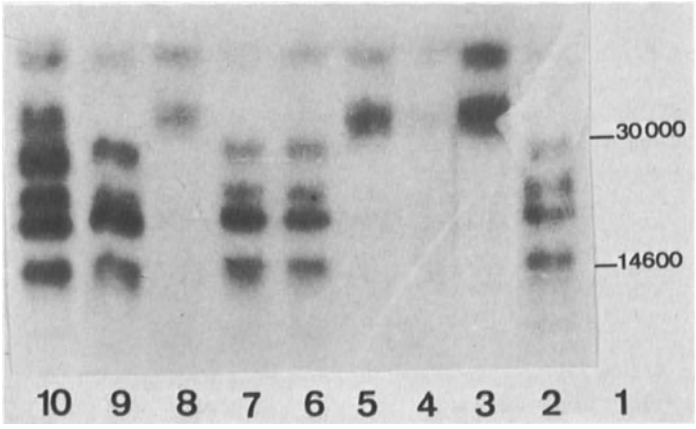

Fig. 1. SDS-PAGE analysis of ^{125}I gp51 peptide fragments generated by limited urokinase digestion and precipitated by the individual monoclonal antibodies. *Lane 1,* molecular weight markers; *lane 2,* peptide fragments precipitated by BC14 (site G); *lane 3,* peptide fragments precipitated by antibodies GA2 and GB10 (site BB′); *lane 4,* peptide by antibodies GB8 (site C); *lane 5,* peptide fragments precipitated by antibodies GA1, GB9, and GB11 (site A); *lane 6,* peptide fragments precipitated by antibodies 6A3 and GB13 (site H); *lane 7,* peptide fragments precipitated by antibodies GA4 (site F); *lane 8,* peptide fragments precipitated by antibodies GA5 and GB15 (site DD′); *lane 9,* peptide fragments precipitated by antibodies GA6, GA7, and GA12 (site E); *lane 10,* unfractionated gp51 peptide fragments

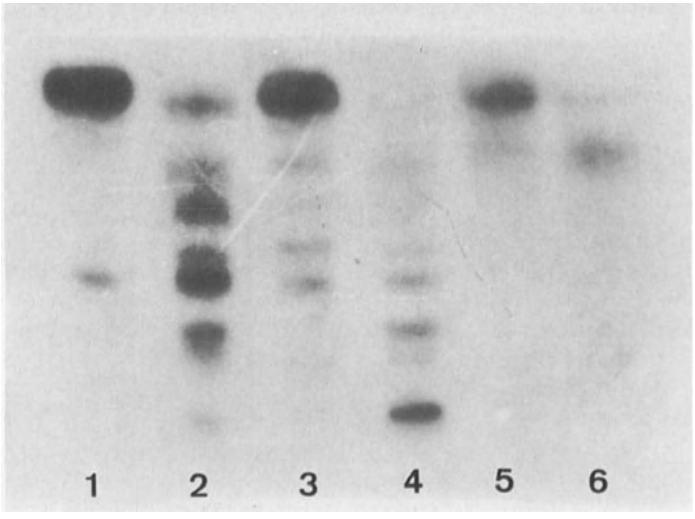

Fig. 2. SDS-PAGE analysis of gp51 peptide fragments generated by limited urokinase digestion. *Lane 1,* undigested ^{125}I-labeled gp51; *lane 2,* UK digested ^{125}I-labeled gp51; *lane 3,* undigested lysyl-specific ^{3}H-labeled gp51; *lane 4,* UK digested lysyl-specific ^{3}H-labeled gp51; *lane 5,* undigested, galactose-specific ^{3}H-labeled gp51; *lane 6,* UK digested, galactose-specific ^{3}H-labeled gp51

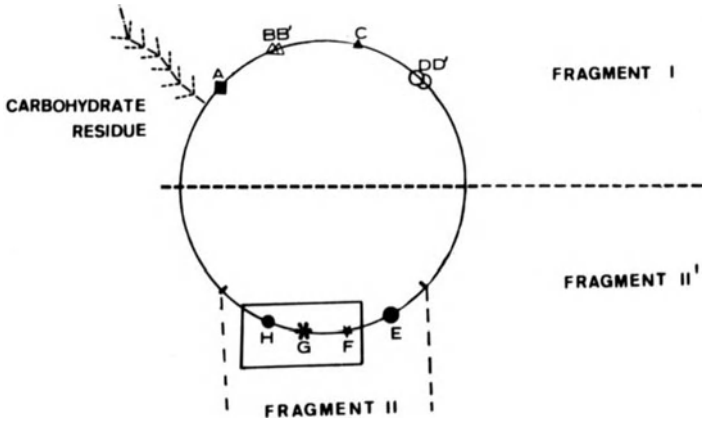

Fig. 3. Model for the location of the epitopes recognized by mouse monoclonal antibodies on the gp51 molecule

Galactose-specific labeling of gp51 followed by urokinase digestion and SDS-PAGE analysis revealed that fragment I of mol. wt. 35,000 contains the major carbohydrate residues of the molecule (Fig. 2). This enabled us to establish a model for the location of the different epitopes at the surface of the gp51 molecule (Fig. 3). In order to identify the epitopes of gp51 involved in virus neutralization and cytotoxicity toward virus-producing cells, we tested the 15 antibodies for:

1. Pseudotype inhibition activity according to Zavada et al. [20].
2. Early polykaryocytosis inhibition activity according to Guillemain et al. [21].

Furthermore, the monoclonal Ab BC14, of Igb2a subclass, was tested for its complement-dependent cytotoxicity toward BLV-producing cells according to Portetelle et al. [16].

The results showed that three of the four epitopes located on the 15,000 mol. wt. nonglycosylated fragment are involved in virus neutralization, and that at least one of these epitopes is also a target for cytotoxic antibodies on virus-producing cells (see Table 1, Fig. 3). Antibodies against fragment I, which contains the major carbohydrate residues of the molecule, displayed none of the biological activities tested for.

C. Discussion and Conclusion

A detailed knowledge of the different antigenic regions of the gp51 molecule is necessary for the development of a BLV

vaccine. In an efficient vaccine, the antigenic regions eliciting a biologically active (virus-neutralizing and cytotoxic) response, must be fully represented in a native, undamaged form. Our monoclonal anti-gp51 antibodies have made possible the mapping of the biologically active subregion on the antigenic molecule.

The results show that the major carbohydrate chains are located on fragment I, which does not seem to contain virus-neutralizing sites. The reactivity of bovine sera (which is abolished when gp51 is deglycosylated) is mostly directed against fragment II, since they display a strong virus-neutralizing activity. The immediate conclusion would be that the involvement of carbohydrates in the antigenic sites recognized by sera of infected cattle is indirect rather than direct, and that removal of carbohydrate chains changes the three-dimensional structure of the molecule and can influence a distal antigenic site. However, the existence on fragment II of small carbohydrate core structures devoid of galactoside residues, which would account for the antigenic properties of gp51 in cattle, cannot be excluded yet.

Our results show that only a fragment of the gp51 molecule is involved in fixation to the cellular receptor, i.e., infectivity. The glycosylated fragment of 35,000 mol. wt. might play a role in providing proper exposure of the active site on the viral particle. BLV-neutralizing monoclonal antibodies can be used as probes for the detection of these important epitopes in the development of a BLV vaccine.

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The excellent technical assistance of Y. Cleuter and M. Leclercq was greatly appreciated.

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Detection and Localization of a Phosphotyrosine-Containing *onc* Gene Product in Feline Tumor Cells*

A. P. Chen, M. Essex, and F. de Noronha

A. Abstract

Protein phosphorylation by a tyrosine-specific kinase is now recognized as a common event in retrovirus-transformed cells. We report in this communication that the feline sarcoma virus (FeSV) encoded transformation-specific proteins (*gag-fes* fusion proteins) and their associated protein kinases are also found in the FeSV in vivo induced tumor preparations, either in the form of fresh tumor homogenate or in the form of cultured cells. With the combined use of subcellular fractionation and detergent extraction we found that the protein kinase activity was present in both the membrane fraction (P100) and the cytosol (S100). The *gag-fes* proteins of two different strains of FeSV were found to associate with the cell framework to different degrees, suggesting that the specific conformational presentation of these proteins may be dictated by the unique portion of each polyprotein. The same *gag-fes* transformation related proteins could be immunoprecipitated with antiserum to phosphotyrosine.

B. Introduction

Retroviruses that transform cultured fibroblasts usually contain an oncogene which is believed to encode for a protein kinase activity [10]. Such oncogenes are highly conserved across species barriers [4]. As assayed in cells transformed in vitro, such putative oncogene products generally have kinase activity that autophosphorylates and/or heterophosphorylates specifically at tyrosine residues [17]. Tyrosine-specific kinase activities have also been found associated with such diverse proteins as the middle T antigen of polyoma virus [24] and the epidermal growth factor-receptor molecule [30]. Despite the rapidly accumulating information on the function of this class of molecule in cultured cells transformed in vitro by different agents, relatively little attention has been given to the study of such proteins in tumor cells. In the current study we examined cells obtained from tumors induced in vivo with feline sarcoma virus (FeSV) for such activities. We also evaluated the practicability of using antisera specific for phosphotyrosine to detect such proteins.

C. FeSV-Specific Transformation Proteins

FeSV, a potent tumor-causing agent, induces rapidly proliferating fibrosarcomas in young kittens. FeSV has been isolated from numerous naturally occurring cat fibrosarcomas [15], but only three isolates (Snyder-Theilen-ST, Gardner-Arnstein-GA, and McDonough-SM) have been studied in great detail. These three FeSV isolates share common features in their genomic structure. As is the case with many other replication-defective retroviruses, the FeSV genome consists of a partially deleted *gag* gene, and *onc* gene insert, and a substantially deleted *env* gene. Only the *gag-*

* Supported by U.S. Public health Service Grants CA-13885, CA-30520, and CA-18216 and American Cancer Society Grant PDT 36

Table 1. Presence of *gag-fes* transformation related proteins in preparations derived from feline tumors

Tumor type	No. of cats	Inoculum	Method of detection	*gag-fes* protein
Fibrosarcoma	4	ST-FeSV	Immunoprecipitation: in vivo ^{35}S-methionine or ^{32}P-orthophosphate labeled short-term cultured cells	pp 85
Fibrosarcoma	5	ST-FeSV	In vitro protein kinase assay on fresh tumor homogenate	pp85
Fibrosarcoma	6	GA-FeSV	Immunoprecipitation; in vivo ^{35}S-methionine labeled cultured cells	p110
Melanoma	3	GA-FeSV	Immunoprecipitation; in vivo ^{35}S-methionine and ^{32}P-orthosphosphate labeled short-term cultured cells	pp110
Melanoma Fibrosarcoma Neurofibrosarcoma Osteosarcoma Chondrosarcoma	5	Unknown, but no FeLV exposure history	Immunoprecipitation; in vivo ^{35}S-methionine labeled short-term cultured cells	–

onc region has been shown to be transcriptionally active. The *onc* portion, which represents the transforming gene of FeSV [21, 26], was acquired from the cell sequence [13]. Recent studies by nucleic acid hybridization demonstrated that the *onc* portion of ST-FeSV and GA-FeSV share more than 50% homology. This homology, however, was not found in the specific portion of SM-FeSV [14]. The *onc* portion of ST- and GA-FeSV is now designated as *fes* while that of SM-FeSV is designated *fms*, denoting their distinct cellular origin [9]. The translation product from the *gag-fes* or *gag-fms* is a fusion polyprotein containing the antigenic moieties of p15, p12 and part of p27 from the *gag* protein, and the *fes* or *fms* protein. The sizes of polyproteins as measured from SDS-PAGE are 85,000 daltons, 110,000 daltons, and 180,000 daltons for ST-, GA-, and SM-FeSV, respectively [1].

The *gag-fes* or *gag-fms* polyproteins were detected, using metabolic labeling with ^{35}S-methionine or ^{32}P-orthophosphate, in cells transformed or transfected in vitro with FeSV. Cells from a broad range of species that were transformed with ST-FeSV and/or GA-FeSV, such as mink [29], goat [1], rat [23], cat [7], and mouse [21], were all shown to contain the *gag-fes* polyprotein. In recent studies we found that the same *gag-fes* polyproteins are present in both fresh tissue homogenate prepared from FeSV-induced firbosarcomas (Table 1) and in tumor cells grown for varying periods of time from a few hours to more than a year [8]. While fibrosarcomas arise when FeSV is inoculated subcutaneously or intramuscularly, the intracutaneous inoculation of GA-FeSV causes the preferential development of melanomas [19]. Although these tumors arise from different embryonic germ layers they express the same *gag-fes* polyprotein when induced by the same virus [7].

Anti-*fes* serum was generated by repeatedly immunizing young adult cats with their own cells that were biopsied and transformed in culture with FeSV in a nonproductive manner. After the removal of those antibodies reactive to the virus struc-

tural proteins by extensive adsorption with gradient-purified FeLV, the cat anti-*fes* serum retained specificity for the "*fes*" determinants on both $P85^{gag\text{-}fes}$ and $P110^{gag\text{-}fes}$ [7].

D. *Gag-fes* Protein Kinase Activity in Tumor Cells

Gag-fes polyproteins of both p85 and p110 were easily phosphorylated when in vitro protein kinase assays were carried out with the respective immunoprecipitates [2, 5, 28]. The *gag-fes* protein-associated kinase seems to autophosphorylate the *gag-fes* molecules and also to be capable of phosphorylating the heavy chain of the immune IgG. The *gag-fes* protein associated kinase prefers the manganese cation (Mn^{2+}) to the magnesium cation (Mg^{2+}) and has an optimal pH for enzymatic reaction of about 7.0 [28]. By phosphoamino acid analysis, the in vivo phosphorylated *gag-fes* polyproteins contain phosphotyrosine as well as phosphoserine residues. The phosphoserine appears to reside in the p12 moiety of the *gag* protein [5]. When analyzed by in vitro phosphorylation, the molecule contains primarily phosphotyrosine.

To determine whether or not the protein kinase activity was also found in tumor cells, as opposed to transformed fibroblasts, we examined preparations from FeSV-induced fibrosarcomas. These preparations included fresh tumor homogenates, tumor cells grown for less than 1 day in culture, and long-term cell lines that were originally established from FeSV-induced tumors. Fresh tissue homogenates were examined

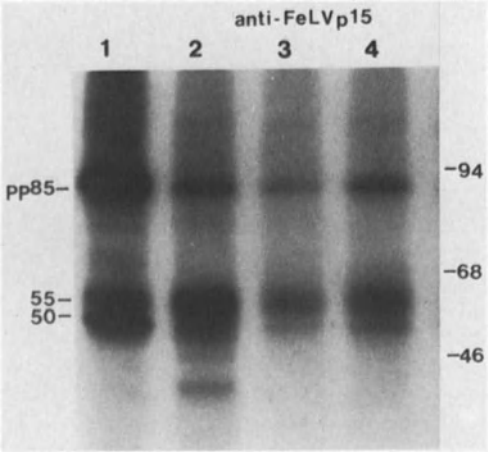

Fig. 1. The *gag-fes* polyproteins prepared from fresh cat tumors are phosphorylated in the in vitro protein kinase assay. ST-FeSV-induced fibrosarcomas in young kittens were examined. The fresh fibrosarcoma tissues were minced in cold Hanks balanced salt solution immediately after excision. The minced tissues were then washed with cold PBS and once with washing buffer containing 0.02 *M* Tris-HCl, pH 6.8, 0.137 *M* NaCl, 0.001 *M* $CaCl_2$, 99 kallikrein inhibitor unit/ml aprotinin, and 1 m*M* phenylmethylsuflonyl fluoride. Subsequently, the minced tumor tissue was resuspended in the washing buffer supplemented with 1% (v/v) NP40 and 10% glycerol and then subjected to homogenization in the Sorvall Omnimix at speed 6 for 3 min (on ice). The final homogenate was clarified once at low-speed centrifugation and then at 100,000 g for 30 min. The clarified lysates were then processed for in vitro protein kinase assay [24]. Three FeLV-positive fibrosarcomas from two kittens were examined in this experiment. Lanes 2 and 4 were prepared from two primary fibrosarcomas that occurred on the site of inoculation. Lane 3 was obtained from a secondary fibrosarcoma or metastatic tumors that appeared at a site distant from the original inoculation. Lane 1 was prepared from cat fibroblasts transformed in vitro with ST-FeSV. Goat anti-FeLV p15 serum was used to immunoprecipitate the *gag-fes* polyproteins. PP85 represents the phosphorylated *gag-fes* protein encoded by ST-FeSV. Fifty-five depicts a phosphorylated protein that comigrates with the IgG heavy chain (superimposed with Coomassie blue stained IgG heavy chain). Fifty is another phoshorylated protein. The molecular weight standards are ^{14}C-labeled phosphorylase b (94,000), bovine serum albumin (68,000), and ovalbumin (46,000)

from five different tumors induced with ST-FeSV, and all were positive for the appropriate kinase activity (Fig. 1). Three proteins were found to be phosphorylated: the $P85^{gag\text{-}fes}$, the heavy chain of immune IgG, and a 50,000-dalton protein of unknown identity.

$P85^{gag\text{-}fes}$ is apparently the most preferred acceptor molecule for this enzymatic reaction. Phosphorylation of the tyrosine residue was confirmed by phosphoamino acid analysis (Fig. 2). Phosphoproteins prepared from this in vitro kinase reaction often also contain low levels of phosphoserine, but it is possible that the latter could be due to a contaminating cellular kinase present in the immunoprecipitation. Similar results have been observed by other investigators [5]. In the phosphorylated proteins recovered from both tumor preparations and in vitro transformed cells, the tyrosine is always the most intensely labeled amino acid. It thus seems apparent that the *gag-fes* protein-associated protein kinase exists in its active form in the ST- and GA-FeSV induced tumors.

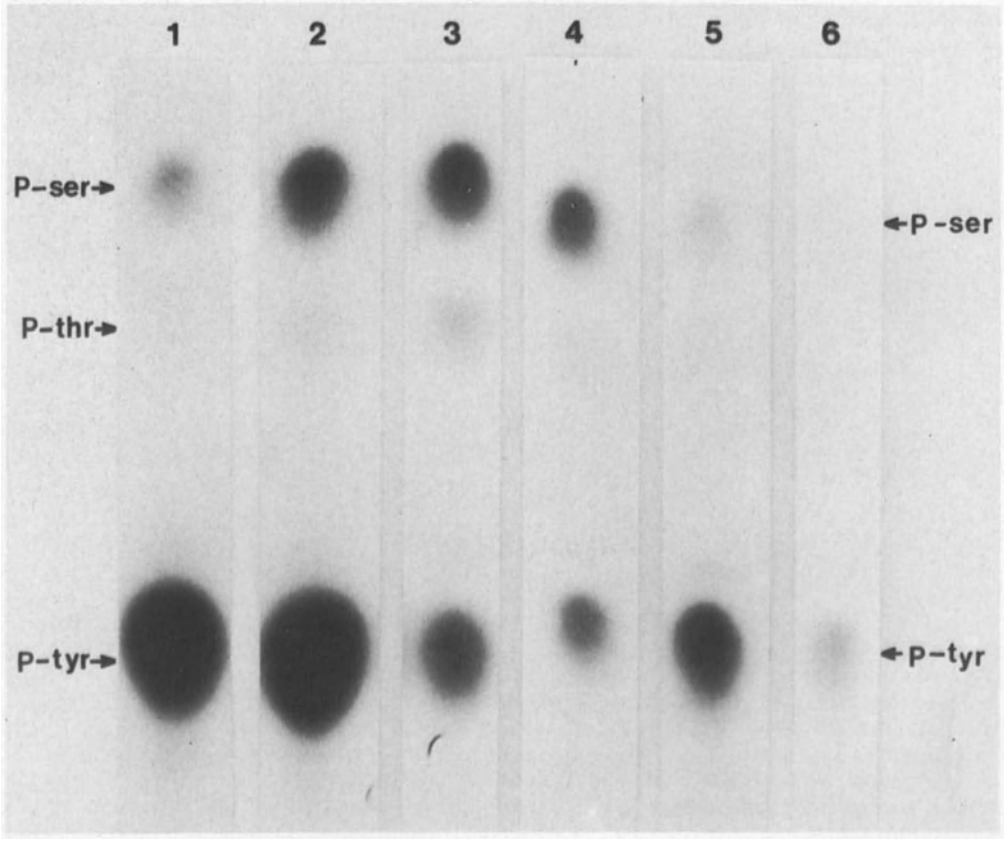

Fig. 2. Phosphoamino acid composition of in vitro labeled phosphoproteins. Phosphoproteins obtained by the in vitro protein kinase assay were electroeluted and hdrolyzed in 6 *N* HCl for 2 h at 110 °C according to Beemon et al. [3]. Phosphoamino acids were separated by one dimensional paper electrophoresis in pyridine-acetic acid (0.5 : 5, at pH 3.5) at 2000 V for 2 h. Markers for phosphoserine (*p-ser*), phosphothreonine (*p-thr*), and phosphotyrosine (*p-tyr*) were located by ninhydrin staining. The phosphoproteins analyzed, and approximate radioactive cpm were: *1*, pp85 from a primary culture of an ST-FeSV induced fibrosarcoma, 4800; *2*, pp110 from GA-FeSV transformed fibroblasts, 6700; *3*, pp110 from a cultured GA-FeSV induced melanoma, 2020; *4*, IgG heavy chain from a GA-FeSV induced melanoma, 1300; *5*, IgG heavy chain from a primary culture of a ST-FeSV induced fibrosarcoma, 1090; *6*, pp50 from the same culture as in *1* and *5*, 410

E. Cellular Localization of the Tyrosine Kinase Activity

Studies with transformation-defective (*td*) mutants of several transforming retroviruses showed that the ability of such viruses to transform cells was closely correlated with the presence of tyrosine-specific protein kinase activity [5, 20, 27]. However, the subcellular localization or cellular compartmentalization of this enzyme activity has not been conlusively established. To address this issue with cells containing the *gag-fes* protein kinase two

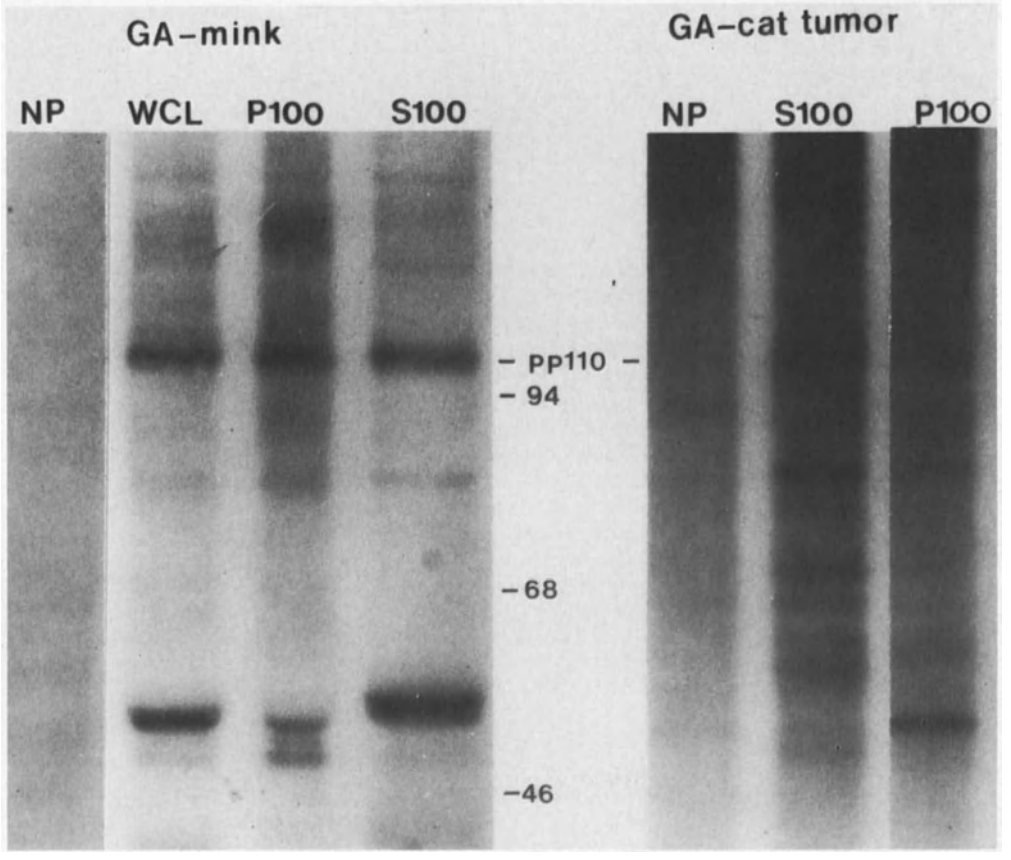

Fig. 3. Subcellular localization of *gag-fes* polyproteins from GA-FeSV induced cat tumor cells and in vitro transformed cells. The protocol for cell fractionation was modified from Hay [16] and Courtneidge et al. [12]. Briefly, cells were collected by scraping into cold PBS and spun at 1000 g for 5 min. Washed cells were resuspended in 5 m*M* KC1, 1 m*M* $MgCl_2$, and 20 m*M* Hepes, at pH 7.0 for 40 min on ice. Cells treated with the hypotonic buffer were homogenized with 20–50 strokes in a tight-fitting Dounce homogenizer, which resulted in more than 90% disruption of cells without apparent damage to the nuclei as examined under the phase-contrast microscope. The clear supernatant was obtained by repeated low-speed centrifugation to remove nuclei and partially broken cells. The clear supernatant was spun at 100,000 g for 45 min to separate the supernatant (S100) and pellet (P100). S100 represents the cytosol fraction while P100 is the crude membrane fraction. The nuclear pellet was washed once with 20 m*M* KC1, 20 m*M* NaCl, 0.1 m*M* EDTA, and 20 m*M* PIPES at pH 6.8, resuspended in 20 ml of 60% (w/w) sucrose in the above washing buffer, overlaid on a 10-ml 60% sucrose cushion and spun at 50,000 g for 60 min. The final pellet (NP) is 99% composed of nuclei and is free of visible contamination with membranes or broken cells as checked in the phase-contrast microscope. WCL is whole cell lysate extracted with NP40-containing lysing buffer. All the fractions were processed by the in vitro protein kinase assay. Goat anti-FeSV p15 serum was used throughout the whole experiment. *GA mink*, GA-FeSV transformed mink cells; *GA-cat tumor*, GA-FeSV induced cat melanoma cells

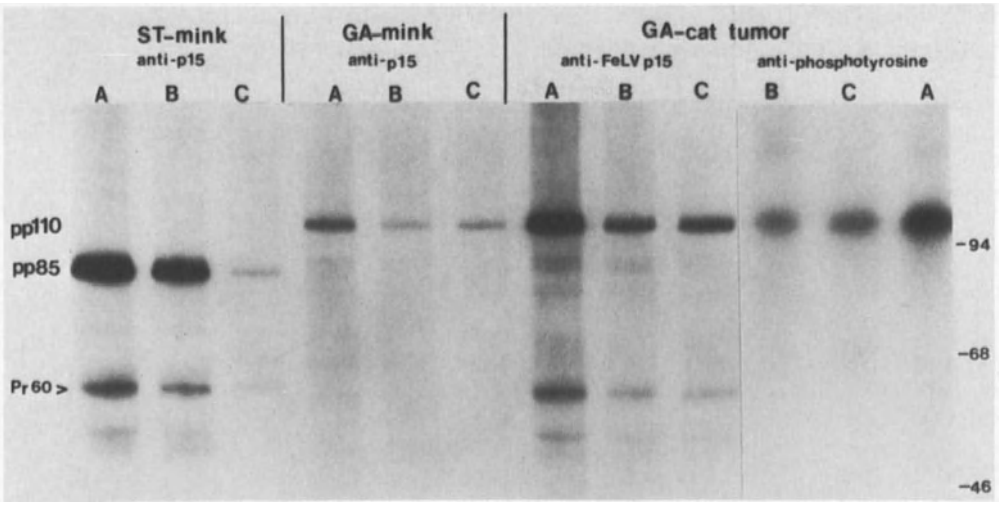

Fig. 4A. Immunoprecipitation of phosphorylated *gag-fes* polyproteins from the soluble fraction and the cytoskeletal fraction. Cells were labeled with 200 μCi/ml of ^{32}P-orthophosphate in phosphate-free Dulbecco-MEM supplemented with 10% dialyzed fetal calf serum for 2 h. The monolayers were then washed four times with cold PBS and twice with cell framework washing buffer containing 0.01 *M* PIPES, pH 6.8, 0.1 *M* KCl, 0.3 *M* sucrose, 0.0025 *M* $MgCl_2$, and 0.001 *M* $CaCl_2$. Subsequently, monolayers were treated with washing buffer containing 1% Triton X-100 at 4 °C for 1 min. The supernatant was collected as the Triton-soluble fraction (lane B). The remaining monolayer was washed twice with washing buffer and immediately lysed with NP40-containing lysing buffer (0.02 *M* Tris-HCl, pH 6.8, 0.137 *M* NaCl, 0.001 *M* $MgCl_2$, 0.001 *M* $CaCl_2$, 10% glycerol, and 1% NP40). The lysate was collected as the Triton-insoluble fraction (lane C). As a positive control, whole cell lysate prepared from NP40 extraction was also included (lane A). All the lysate preparations were clarified at 100,000 g for 20 min and were preclarified with normal serum before immunoprecipitation with goat anti-FeLV p15 (5 μl) or guinea pig antiphosphotyrosine serum (2 μl). The cells examined were ST-FeSV transformed mink cells (*ST-mink*), GA-FeSV transformed mink cells (*GA-mink*) and GA-FeSV induced cat melanoma cells (*GA-cat tumor*). *Pp110* is a phosphorylated *gag-fes* polyprotein encoded by GA-FeSV, *pp85* is *gag-fes* encoded by ST-FeSV, and *Pr60* represents the phosphorylated precursor molecule of *gag*. The molecular weight markers are labeled on the *right*

approaches were used: subcellular fractionation and the in situ labeling of cells from the exterior.

Cells were fractionated into a soluble fraction (S100), a crude membrane fraction (P100), and a nuclear pellet (NP) according to Hay et al. [16] and Courtneidge et al. [12]. In vitro protein kinase activity was detected in both the soluble fraction and the membrane fraction of both transformed fibroblasts and the tumor cells (Fig. 3). A nonionic detergent, Triton X-100, when used at low concentrations (0.5%–1%), extracts approximately 80% of the total cell proteins, leaving 20% with the cytoskeleton [6]. This detergent appeared to disrupt the plasma membrane and release the cell contents without rearranging the Triton cytoskeleton.

As shown in Fig. 4A, more than 80% of the in vivo labeled pp85 was extracted in the Triton-soluble fraction (lane B) while less than one-fifth was tightly associated with the Triton cytoskeleton (the insoluble fraction – lane C) in ST-FeSV transformed mink cells. In contrast, equal amounts of pp110 from the GA-FeSV induced tumor cells and the GA-FeSV transformed cells were found in Triton-soluble and insoluble fractions. The profile of in vitro kinase activity correspond to the concentration of phosphorylated *gag-fes* proteins found in each fraction. For example, the Triton-soluble fraction of ST-mink cells contained most of the in vivo phosphorylated pp85 (lane B of Fig. 4A) as well as the majority of the protein kinase activity (lane A of Fig. 4B). An analogous pattern was found

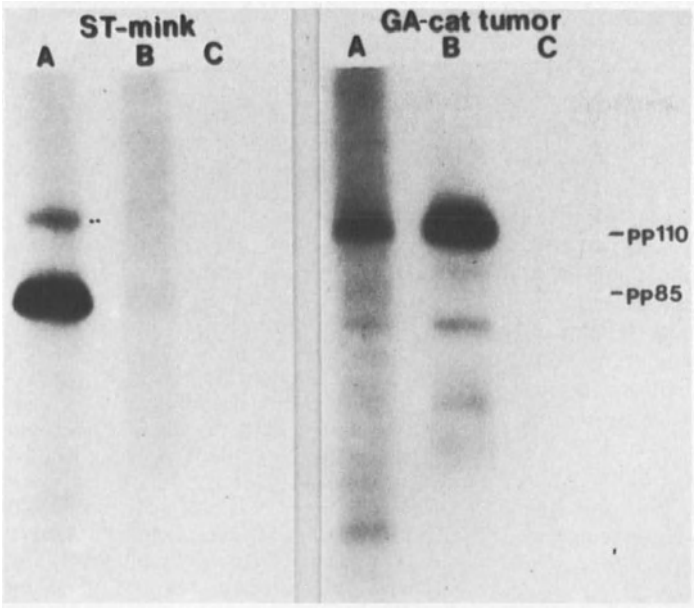

Fig. 4B. Phosphorylation of *gag-fes* polyproteins in situ compared with phosphorylation in the soluble fraction. Cells were grown to a density of 3×10^6 cells per 6.0-cm tissue culture dish, and washed as described in the legend of Fig. 4A. The cells were either treated with Triton X-100 (1%) in washing buffer for 1 min or untreated. The supernatant from Triton X-100 treated cells was saved as the Triton-soluble fraction (lane A) for the in vitro protein kinase assay. Both Triton-pretreated (lane B) and untreated (lane C) culture dishes were incubated with 250 μCi γ-^{32}P-ATP (ICN, Irving, CA) and 2×10^{-5} *M* ATP in the cell framework washing buffer at room temperature for 15 min. The cell monolayers were then carefully washed with cold PBS washing buffer and finally were lysed in 0.5 ml of NP40-lysing buffer. Five microliters of goat anti-FeLV p15 serum was used in the immunoprecipitation. The results obtained with ST-FeSV transformed mink cells (*ST-mink*) and GA-FeSV induced melanoma cells (*GA-cat tumor*) are presented. The nonspecific reactivity of the ST-mink cell preparation of goat serum is indicated by dots (. .). *Pp110* and *pp85* are the phosphorylated *gag-fes* polyproteins of GA-FeSV and ST-FeSV, respectively

for GA-FeSV transformed cells as observed in both in vivo and in vitro assays (compare Fig. 4A and 4B).

Attempts to label the cellular protein with exogenously supplied γ-^{32}P-ATP from the cell exterior were unsuccessful (lane C of Fig. 4B), suggesting that the active site of this kinase is not exposed to the exterior of cells and that the plasma membrane is not permeable to γ-^{32}P-ATP. Treatment of the cells for 1 min with Triton X-100 allows the entry of γ-^{32}P-ATP to encounter those cytoskeleton-associated proteins and to allow the active transfer of phosphorus to the substrate, P110$^{gag\text{-}fes}$ (lane B of GA tumor in Fig. 4B). Such activity was not detected in ST-FeSV transformed cells, since more than 80% of the pp85 was extracted with Triton X-100 in the soluble fraction (lane B of ST mink in Fig. 4B).

Our results indicate that the *gag-fes* polyproteins are found in both cytosol and membrane fractions and are linked to the cell framework. It seems likely that there are two pools of *gag-fes* proteins: one membrane-bound, and the other cytoskeleton associated and subsequently fractionated in the cytosol (S100). Alternatively, it seems possible that the *gag-fes* polyprotein might be anchored partly in the cytoplasmic side of the plasma membrane and partly with the cytoskeleton. Our current evidence, however, cannot distinguish between these two possibilities.

F. Detection of Transformation Proteins Using Antisera to Phosphotyrosine

As mentioned earlier, phosphorylation at tyrosine residues appears associated primarily with transformation-related proteins. Some phosphotyrosine-containing proteins represent the autophosphorylated enzyme molecule itself, while others represent an independent substrate species [3, 11, 18]. Because of the unusual transformation-associated nature of the phosphotyrosine reaction and the relative conservation of the retrovirus-encoded genes which mediate this activity, we reasoned that an antiserum directed specifically to phosphotyrosine might be a useful tool for the detection of other proteins of this same class. in a previous attempt of this type, Ross et al. [22] used an antiserum to azobenzyl phosphonate to detect the pp120 of cells transformed by the Abelson murine leukemia virus and also a previously undescribed protein designated pp110 which was found in mouse fibroblasts tranformed with the Schmidt-Ruppin strain of Rous sarcoma virus.

We used antiserum made against phosphotyrosine coupled to bovine gamma globulin by means of the carbodiimide method (a generous gift from Drs. Schaffhausen and Benjamin, Harvard Medical School). the antiphosphotyrosine serum specifically interacts with the in vivo ^{32}P-labeled pp85$^{gag\text{-}fes}$ and pp110$^{gag\text{-}fes}$ of FeSV as well as with the middle T antigen of polyoma virus (Schaffhausen and Benjamin, personal communication). A specificity control for this serum is shown in Fig. 4A. Antiphosphotyrosine-containing serum recognized only the pp110$^{gag\text{-}fes}$ but not the phosphoserine-containing precursor molecule of the gag protein (Pr60). The antiphosphotyrosine also immunoprecipitated several other proteins at molecular weights of approximately 50,000 daltons and 140,000 daltons (data not shown). Our preliminary results demonstrated the potential of this specific antiserum. Subsequent applications may facilitate the identification of phosphoproteins in the context of either distinct substrate(s) for transforming proteins or kinase molecules themselves which have autophosphorylating potential.

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The Use of cDNA Cloning Techniques to Isolate Genes Activated in Tumour Cells *

M. R. D. Scott, P. M. Brickell, D. S. Latchman, D. Murphy, K.-H. Westphal, and P. W. J. Rigby

A. Introduction

Oncogenic transformation by tumour viruses requires, in many cases, the function of only one viral gene. In the case of the papovavirus Simian virus 40 (SV40) it is the gene coding for large T-antigen [8, 16] while for most strongly transforming retroviruses it is a gene of cellular origin [1]. Recent work has shown that many murine and human tumours, for which there is no indication of viral involvement, contain genes which have been altered in such a way that they have acquired the ability to transform the NIH3T3 line of mouse cells [3, 5, 7, 10, 15]. In order to understand the molecular mechanisms of oncogenesis it is necessary to analyse in detail the biochemical functions of the protein products of these various types of transforming gene. However, even a total description of the activities of a transforming protein will not reveal the complete mechanism of oncogenesis. Transformed cells differ from their normal parents in a multitude of biological and biochemical properties and it is unlikely that all of these changes occur as a direct result of the action of the transforming protein. Rather, the product of the oncogene must reprogramme the cell's metabolism and/or gene expression so that having defined the transforming protein it is then necessary to identify its cellular targets.

The large T-antigen protein encoded by the early region of the SV40 genome is both necessary and sufficient for morphological transformation in vitro and for tumorigenesis in vivo [8, 16]. However, this protein possesses an extraordinary variety of biochemical activities [i.e. ref. 13], many of which are poorly understood. It binds with high affinity to the viral origin of DNA replication in a reaction required for the initiation of viral DNA replication. It also binds with lower affinity to cellular DNA and is capable of inducing a round of DNA replication in quiescent cells. The protein is displayed on the surface of transformed cells, although the vast majority of the antigen is nuclear, it has an ATPase activity and it can overcome the block to the productive infection of monkey cells by human adenoviruses. Large T-antigen represses its own synthesis by binding to the promoter of the viral early transcription unit and there is evidence that it can affect the transcription of cellular genes. Which of these activities are directly involved in transformation is not known.

There is evidence from solution hybridisation experiments that the cytoplasmic mRNA populations of cells transformed by SV40 or by Rous sarcoma virus (RSV) can be distinguished from those of their normal parents [6, 17]. SV40 provides a particularly attractive system in which to investigate this problem because the viral transforming protein has the properties expected of a transcriptional regulator. Two lines of evidence suggest that large T-antigen can di-

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rectly modulate the transcription of cellular genes. The first detectable event following the synthesis of large T-antigen in infected permissive or non-permissive cells is the induction of several cellular enzymes involved in nucleotide metabolism and DNA replication, for example, thymidine kinase [16]. Although there is no evidence that this induction is transcriptional, this seems to be a reasonable proposition. More directly, Baserga and his colleagues have shown that in human-mouse somatic cell hybrids only one of the two rRNA gene complements is active. Introduction of large T-antigen into such cells activates the previously quiescent rRNA gene complement at the level of transcription [14]. We therefore decided to seek to identify and isolate cellular genes which are switched on or off in SV40-transformed mouse cells.

B. Results

Our approach has been to use cDNA cloning techniques to construct libraries of plasmid clones representative of the polyadenylated, cytoplasmic mRNA populations of normal and transformed cells and then to screen these libraries for genes expressed at different levels in the two cell types. Such libraries must be truly representative and we have therefore developed techniques which produce cDNA clones containing large inserts at very high efficiency, 2×10^5 clones per microgram polyadenylated RNA. We constructed two such libraries, one from the normal Balb/c 3T3 mouse cell line and one from an SV40-transformed derivative SV3T3 C138 [2, 11, 12]. The libraries were plated at high colony density and then replica filters were

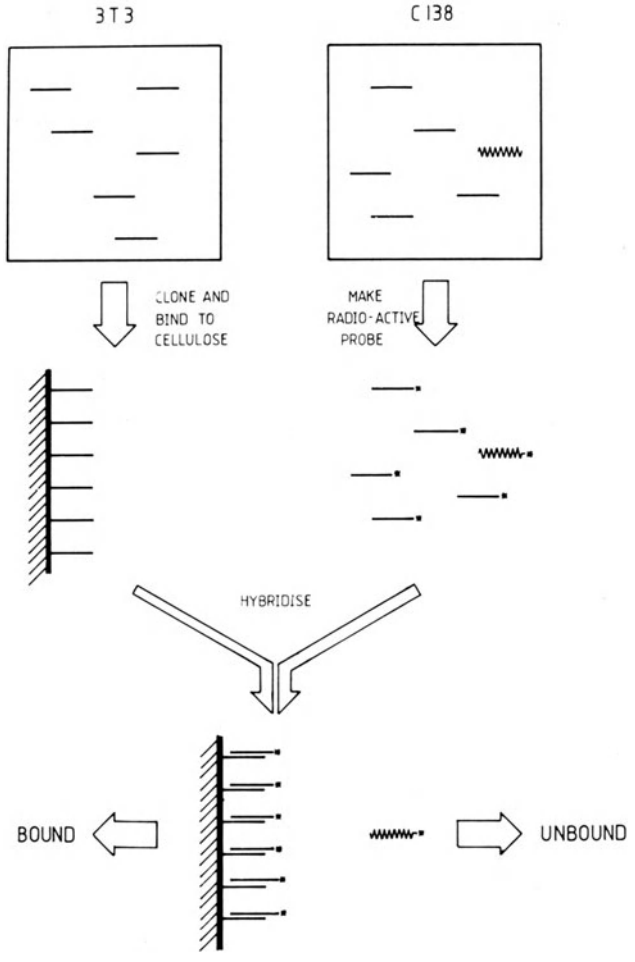

Fig. 1. Procedure for preparing a cDNA probe specific for genes expressed at higher levels in tumour cells than in normal cells. The Balb/c 3T3 cDNA library was pooled and grown in mass culture; plasmid DNA was isolated and covalently coupled to cellulose [9]. ^{32}P-labelled cDNA was prepared using SV3T3 C138 mRNA as the template and then hybridised to the immobilised normal cell cDNA. Sequences held in common between the two cell types will hybridise; sequences expressed only in the transformed cell will not

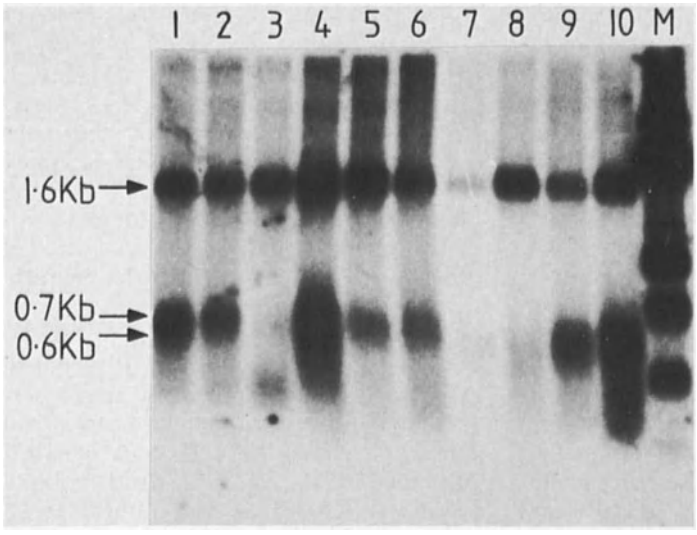

Fig. 2. Transfer hybridisation analysis of mRNAs from mouse cells transformed by a variety of agents. Polyadenylated, cytoplasmic RNA was fractionated by electrophoresis in an agarose gel containing formaldehyde and then transferred to a nitrocellulose filter [4]. The filter was then hybridised with ^{32}P-labelled pAG64, a plasmid containing a cDNA insert which hybridises to mRNAs of 1.6 kb, 0.7 kb and 0.6 kb present at elevated levels in SV3T3 C138 relative to Balb/c 3T3. The tracks are: *1,* Balb/c mouse fibroblasts transformed by methylcholanthrene epoxide; *2,* Balb/c mouse fibroblasts transformed by methylcholanthrene; *3,* NIH3T3 cells transformed by A-MuLV; *4,* a second line of NIH3T3 cells transformed by A-MuLV; *5,* NIH3T3 cells transformed by RSV; *6,* Swiss 3T3 cells transformed by RSV; *7,* normal Balb/c mouse fibroblasts; *8,* Balb/c 3T3 cells transformed by polyoma virus; *9,* a second line of Balb/c 3T3 cells transformed by polyoma virus; *10,* SV3T3 C138. The track labelled *M* contains DNA size markers

prepared and screened by hybridisation with labeled mRNA from the two cell types. In such a colony screening the intensity of the autoradiographic signal is a measure of the abundance of a particular sequence within the total RNA population and we could thus readily search for clones corresponding to mRNAs present at higher or lower levels in the transformed cell line. However, this approach failed to reveal any differentially hybridising colonies. Such colony screening detects only those RNAs of an abundance of 0.1% or above. Our data therefore indicate, in agreement with those of Williams et al. [17], that despite their very different biological and biochemical properties normal and transformed cells do not differ in their abundant mRNAs.

We remained convinced that there must be differentially expressed genes and therefore sought to develop procedures for enriching the corresponding mRNAs. The procedure we adopted is shown in Fig. 1. The Balb/c 3T3 cDNA library was pooled and grown in mass culture; plasmid DNA was then isolated and covalently coupled to a cellulose support. ^{32}P-labelled cDNA was prepared using SV3T3 C138 mRNA as the template and hybridised repeatedly to the immobilised normal cell cDNA. This procedure should remove those RNA sequences held in common between the two cell types and thus the cDNA which fails to hybridise should represent the differentially expressed genes. We analysed the enriched cDNA by hybridising it in solution to vast excesses of either normal or transformed cell mRNA and thus showed that it did indeed detect sequences present in the transformed cell but not in the normal cell. We used this cDNA to screen the SV3T3 C138 cDNA library and thus isolated a number of clones. We have used these cDNA clones as probes in transfer hybridisation experiments [4] to analyse the polyadenylated, cytoplasmic mRNAs of Balb/c 3T3 and SV3T3 C138 cells. Our data con-

firm that the clones correspond to mRNAs present at higher levels in SV40-transformed cells than in normal cells and characterise the relevant transcripts. We have also isolated the corresponding genomic DNA sequences from bacteriophage λ libraries of mouse DNA and are presently determining the detailed structures of these genes and of their RNA products.

It was clearly of interest to ask whether these same genes are activated in mouse cells transformed by agents other than SV40. We have used transfer hybridisation techniques to answer this question and an example of our data is shown in Fig. 2. The plasmid pAG64 contains a cDNA insert which hybridises to three RNAs, of 1.6 kb, 0.7 kb and 0.6 kb, in SV3T3 C138 cells. These RNAs are present in Balb/c 3T3 cells but at a much lower level. Figure 2 shows clearly that the 1.6 kb RNA is also present at a higher abundance in mouse cells transformed by polyoma virus, by RSV, by Abelson murine leukaemia virus (A-MuLV) and by the chemical carcinogens methylcholanthrene and methylcholanthrene epoxide. The 0.7 kb and 0.6 kb RNAs are also present in several of the transformed cell lines.

C. Discussion

Our work has shown that the application of recombinant DNA technology makes it possible to analyse very sensitively the differences in gene expression between normal and tumour cells. We have isolated a number of cDNA clones which correspond to mRNAs present at a higher level in SV40-transformed mouse cells than in their normal parents and we are presently analysing the mechanism by which viral transformation affects the expression of these genes. At least one of these genes is also expressed in fibroblasts transformed by a wide variety of agents including a murine leukaemia virus. The transforming proteins of A-MuLV and RSV are not thought to be capable of interacting directly with the genome and thus it seems likely that there are several mechanisms by which viral transforming proteins can affect the expression of these genes.

The technology that we have developed is clearly applicable to other transformation systems and it should be possible to use it to analyse the differences in gene expression which distinguish leukaemic cells from their normal counterparts. In this way will be able to build up a detailed picture of the changes in gene expression which occur during the induction and progression of leukaemia and thus further our understanding of this disease.

Our studies of the genes we have isolated would be greatly facilitated if we knew their function in a normal mouse. In this regard our recent data are of considerable interest. We have shown that the plasmid pAG64 detects no RNAs in a variety of adult mouse tissues but that there are a large number of transcripts homologous to it in mouse embryos and in cell lines derived from embryos, for example teratocarcinoma cells. These observations raise the interesting possibility that the viral transforming proteins act upon genes normally involved in embryonic development and we are currently exploring this idea in detail.

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Cellular Transforming Genes in Cancer*

M. A. Lane, A. Sainten, D. Neary, D. Becker, and G. M. Cooper

Genes which regulate growth in normal cells during differentiation function in response to appropriate signals which allow proliferation and maturation to proceed in an orderly manner. In the neoplastic cell, genetic control of proliferation appears to be lost. Non-neoplastic cells may contain a number of genes with potential transforming activity which are normally well regulated and only expressed at a particular phase of cell differentiation. Abnormal expression of these genes may result in neoplastic growth. Experiments by Cooper and co-workers have addressed the question of potential transforming activity of genes from normal cells [1]. In this series of experiments, DNA from normal cells was prepared, sheared to a size range of 0.5–5.0 kilobases, and transfected as a calcium phosphate precipitate into NIH 3T3 cells. Transforming efficiences of high molecular weight DNAs from these cells were approximately 3×10^{-4} transformants/μg DNA, while transforming efficiencies for sheared DNAs were tenfold greater. Transforming efficiencies of DNAs from foci isolated in primary transfection of sheared DNA when tested in secondary transfection were 100–1000 fold higher (0.1–1.0 foci/μg DNA), comparable to transforming activities of strongly oncogenic viruses [2, 3]. These studies suggest that normal cell genes, when expressed abnormally, can transform at high efficiencies.

High molecular weight DNA from neoplastic cells, unlike that from normal cells, transforms NIH 3T3 cells in primary transfection with high efficiencies of 0.1–1.0 transformants/μg DNA, suggesting that events at the DNA level have occurred already which have freed these genes from appropriate control. Activated transforming genes which can be efficiently transmitted by transfection with high molecular weight DNAs have been found in chemically transformed mouse fibroblasts [4], B-cell lymphomas and a nephroblastoma induced by avian lymphoid leukosis viruses [5], human bladder carcinomas [6, 7], mammary tumors of mouse and human origin [8], gliomas and neuroblastomas of rat and mouse origin [9], and human colon carcinoma and promyelocytic leukemia [10]. The transforming genes detected by transfection of DNAs of tumors induced by lymphoid leukosis viruses, a class of retroviruses which lack viral transforming genes, and those of mammary tumors associated with mouse mammary tumor virus, which also lacks a viral transforming gene, are not linked to viral DNA sequences, suggesting that oncogenesis by these viruses involves indirect activation of cellular transforming genes [5, 8].

Hayward and co-workers [11] have demonstrated that in LLV-induced tumors, a cellular gene (*c-myc*), homologous to the transforming gene of acute leukemia virus, is activated by adjacent integration of viral DNA (LTR insertion). However, analysis of NIH cells transformed by DNAs of these neoplasms indicates that transformation

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was not mediated by transfer of the *c-myc* gene to the NIH cells [12]. These observations indicate that at least two different cellular genes are activated in LLV-induced neoplasms. As carcinogenesis is a multistage process, activation of *c-myc* may precede or complement activation of the cellular transforming gene detected by transfection. Alternatively, LLV may act at an early preneoplastic stage to expand the population of cells in which transforming events occur, which is consistent with the mechanism proposed by McGrath and Weissman for induction of T-cell lymphomas by analogous retroviruses of the AKR mouse [13]. These findings thus suggest that transformation by a variety of carcinogenic agents can involve dominant mutations or gene rearrangements resulting in the activation of cellular transforming genes which are then detectable by transfection.

Weinberg and co-workers have demonstrated that mouse fibroblasts transformed by several different chemical carcinogens contained related activated transforming genes as determined by restriction enzyme analysis [4]. We have studied a series of mammary tumors of mouse and human origin. In these experiments, high molecular weight DNAs of five mouse mammary tumors, two chemical carcinogen-induced mouse mammary tumors, and one human mammary tumor cell line (MCF-7) were assayed for the presence of transmissible activated transforming genes by transfection. DNAs of six mouse tumors and the human tumor cell line induced transformation with high efficiencies. The transforming activities of DNAs of all five MMTV-induced tumors, the chemical carcinogen-induced mouse tumor, and the human tumor cell line were inactivated by digestion with the restriction endonucleases *Pvu*II and *Sac*I, but not by *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, or *Xho*I. These results suggest that the same transforming gene was activated in six different mouse mammary carcinomas, induced by either MMTV or a chemical carcinogen, and in a human mammary carcinoma cell line [8]. This transforming gene differs by restriction analysis from that activated in two human bladder carcinomas [6] and from the gene activated in chemically transformed fibroblasts [4]. These findings suggest that within neoplasms of a particular differentiated cell type, a common transforming gene is activated. Cloning of the human mammary tumor transforming sequence has recently been completed. Transforming efficiency of this clone is 40,000 foci/μg cell DNA insert, and as expected from our previous studies, the transforming sequence contains both *Sac*I and *Pvu*II sites.

Sera from mice-bearing tumors induced by NIH 3T3 cells transformed by mouse or human mammary tumor DNA immunoprecipitated an 86,000-dalton glycoprotein in extracts of NIH cells transformed by human mammary carcinoma DNA. This antigen was also immunoprecipitated by sera from mice-bearing tumors induced by mouse mammary carcinoma DNAs and from mice-bearing primary mammary carcinomas. These results indicate that this protein represents an antigen which is specifically associated with expression of the transmissible transforming genes of human and mouse mammary carcinomas [14].

Molecular cloning of the transforming gene activated in chicken bursal lymphoma has been carried out by Goubin et al. [15]. Use of this cloned gene as a probe in Southern blotting analysis indicates that this gene is evolutionarily well conserved in that hybridization to human DNAs is of comparable intensity to that observed in hybridization of the probe to chicken DNA. The cloned chicken bursal gene is biologically active and transforms with efficiencies 10^5 times enriched over uncloned bursal tumor cell DNA.

There are several conclusions to be drawn from these studies. We may first assume that cellular transforming genes, in their normal state, are carefully regulated and may only be expressed or "turned on" at a particular phase of cellular differentiation in response to the appropriate external signals. We can speculate that, as a rare event, a dominant genetic change in the control elements of these genes can lead to unregulated growth which results in the production of a neoplasm. Clearly, one or several steps may be involved in the deregulation of such a gene. The end result, however, may be successfully scored by transfection. A second point which emerges is that these genes appear to be well con-

served in evolution, based upon the apparent similarity by restriction enzyme analysis between human and mouse mammary tumor transforming genes [8, 14], and from results obtained with the cloned chicken bursal transforming gene [15]. This suggests that a gene cloned from a neoplasm of mice may share substantial homology with the analogous gene in humans. These studies also demonstrate that within a particular differentiated phenotype, a common transforming gene is activated which may be particular to the differentiated state of the neoplasm.

Considering these data, we have speculated that in a specific cell lineage which undergoes several differentiative steps to maturity, one or several of the genes expressed at these steps may be susceptible to rearrangements or mutations which can lead to neoplastic transformation. To examine this hypothesis more closely, we have chosen a cell lineage where differentiation can be described in terms of migration, surface markers, and cellular function in maturity. Cells of the immune system seem most appropriate for these investigations.

Within the immune system, the differentiative lineages of B- and T-lymphocytes are perhaps the most well characterized. Cell surface markers exist, which allow classification of cell types as early, intermediate, or mature in both human and murine systems. Neoplasms which arise from T- and B-lymphocyte populations have been characterized with regard to their state of differentiation by surface markers, by function in in vitro assays, and, in B-cell neoplasms, by the degree of heavy and light chain production, assembly, and secretion. These neoplasms can be categorized as representative of early, intermediate, or mature counterparts of normal T- and B-lymphocyte differentiation. Identification, characterization, and molecular cloning of cellular transforming genes from T- and B-lymphocyte neoplasms, representative of early, intermediate, and mature stages of normal cellular differentiation, will provide the tools to examine genetic events which lead to neoplastic transformation. These studies will allow us to assess the number of genes at risk of neoplastic transformation within a well-described normal cell differentiative lineage. Probes generated in these experiments may provide new insights into the mechanisms by which growth is regulated in normal lymphocytes and may lead to an understanding of molecular events which signal cellular proliferation and differentiation.

To determine whether transmissible transforming genes could be detected in T- and B-lymphocyte neoplasms by transfection of NIH 3T3 cells, we prepared high molecular weight DNA (> 20 kb) from more than 20 different neoplastic cell lines or primary patient isolates from T- and B-lymphocyte neoplasms of mouse or human origin. Calcium phosphate precipitates of these DNAs were applied to NIH 3T3 cells, and primary transformation efficiencies were scored after 12–14 days of culture [16]. Efficiencies of these DNAs in primary transfection ranged from 0.05 to 0.5 foci/μg DNA. Four to six foci from each primary transfection were picked and grown in mass culture. DNAs were prepared from these foci of NIH cells transformed by tumor DNAs and were used as donor DNAs in secondary transfection assays. Transforming efficiencies in secondary transfection also ranged between 0.05 and 0.5 foci/μg DNA, indicating that transmissible activated transforming genes from the neoplasms were present in the transformed NIH cells. DNA from spontaneous transformants, which occasionally arise in NIH 3T3 cells, does not retransform in secondary transfection assays; thus we were assured that these assays were identifiying only dominant activated transmissible transforming genes in transformants from these neoplasms. Transforming efficiencies for some of the T- and B-neoplasm DNAs tested in primary and secondary transfection assays are presented in Tables 1 and 2. Efficient transformation of NIH cells were achieved with DNAs from frozen patient cells, primary tumors, or cell lines of both mouse and human origin. From this table it may be also observed that from B-lymphocyte lineage neoplasms we have obtained transformation using DNA from tumors representative of early, intermediate, and mature stages of normal differentiation. From neoplasms of the T-lymphocyte lineage we have identified transformants representative of intermediate

Pre-T (?) neoplasm	Intermediate T neoplasm	Mature T neoplasm
Human None	*Human* (1) T10[a]	*Human* (1) Sezary Syndrome[a]
Mouse (2) SJL Transforming efficiency = 0.15 foci/μg DNA	*Mouse* (1) S49 (1) W7.1 (1) L691 (1) KKT2 (1) SL3 (1) SL7 Transforming efficiency = 0.10 – 1.0 foci/μg DNA	*Mouse* (1) Clone A (1) 104.6 Transforming efficiency = 0.05 – 0.55 foci/μg DNA
	Controls	
	Normal mouse thymocytes	< 0.003
	Helper clone 101.6	< 0.001
	Human embryo fibroblasts	< 0.002
	Salmon sperm	< 0.005

Table 1. T-lymphocyte neoplasms possessing transmissible transforming genes

Cell lines or patient isolates designated (1) are described by Lane et al. [16]. Cell line designated (2) was obtained from M. Schied

[a] Indicates primary tumor

and mature stages of normal differentiation. Because of a lack of cell surface markers to characterize prethymic tumors, obtaining a representative for this classification has proved difficult. At present, we have several candidate neoplasms which show no heavy chain gene rearrangement, have no theta on their surface, and are terminal transferable inducible. Assays are currently in progress to determine whether DNAs from these tumors will transform NIH 3T3 cells in culture. With the completion of these studies, we will have identified transmissible transforming genes from T- and B-lymphocyte neoplasms which represent early, intermediate, and mature stages of normal differentiation, and will have completed our first goal in these studies.

Restriction enzyme analysis of transforming genes has proved useful in two ways. First, it has allowed us to demonstrate that activation of a specific transforming gene is correlated with a particular differentiated cell type in neoplasms representative of that particular stage of normal differentiation. Second, identification of restriction enzymes which do not inactivate transforming genes provides a useful cloning strategy by means of a transforming gene enrichment step.

To carry out this type of analysis, we selected four six base recognition restriction endonucleases which cleave cellular DNA statistically once every 4 kb. We chose *Eco*RI, *Hind*III, *Bam*HI, and *Xho*I to cleave whole cell DNA containing transforming genes of interest. Digestions were monitored by gel electrophoresis to assure that complete digestion had occurred. Both digested DNAs and companion undigested samples were transfected onto NIH 3T3 cells, and foci were enumerated. If a transforming gene possessed the six nucleotide base sequence recognized by the restriction endonuclease, the gene would be cleaved. Cleavage within the transforming gene sequence resulted in a reduction in transformation efficiencies by direct inactivation of the transforming gene. In this manner, a "finger print" of the transforming gene could be generated based upon enzyme inactivation patterns.

These findings are summarized in Table 3. We are currently continuing and expanding these. To date, by this method of

Table 2. B-lymphocyte neoplasms possessing transmissible transforming genes

Pre-B neoplasms	Int. B neoplasms	Mature B neoplasms
Human	*Human*	*Human*
(1) $C^+B^+1^a$	(5) Raji	(1) GM1500
(1) $C^+B^+2^a$	(5) Namalwa	(1) GM2132
(1) $C^-B^-1^a$	(5) BJAB	*Mouse*
(1) $C^-B^-2^a$	(6) MC116	(1) S107
(1) 207	(6) EW36	(1) M315
(1) 697	(6) CW678	(1) NS2.1
(2) SMS-SB	*Mouse*	Transforming efficiency 0.11 – 0.20 foci-μg DNA
(3) NALM-1	(1) W231	
Mouse	(1) 2PK3	
(4) B6T4E4	(1) BCL-1	
(4) B6T1E1	Transforming efficiency 0.15 – 1.2 foci/μg DNA	
Transforming efficiency 0.09 – 1.10 foci/μg DNA		

	Controls	
	EBV immortalized B-lymphocytes (2)	< 0.005
	Hu emb. fibroblasts	< 0.002
	Salmon sperm	< 0.005

Cell lines or patient isolates designated (1) are described by Lane et al. [16]. (2) Human pre-B cell line prepared by G. Smith and B. Ozan manuscript in preparation. (3) Blood, Vol. 58:648 (1981). (4) Mouse Ablesome-induced pre-B tumors, C57BL/6 strain from R. Risser. (5) African Burkitts patient cell lines from I. McGrath. (6) American Burkitts cell lines from I. McGrath

[a] Indicates primary tumor

Table 3. Restriction analysis of B-lymphocyte neoplasm transforming sequences

	*Eco*RI	*Bam*HI	*Xho*I	*Hind*III	*Sac*I
Pre-B neoplasms					
Six human	–	+	+	–	ND
Two mouse	–	+	+	–	ND
Intermediate B neoplasms					
Two human	–	+	–	–	ND
Three mouse	–	+	–	–	ND
Mature B neoplasms					
Two human	–	–	–	–	+
Two mouse	–	–	–	–	+

Restriction analysis of T-lymphocyte neoplasm-transforming sequences

	*Eco*RI	*Bam*HI	*Xho*I	*Hind*III
Intermediate T neoplasms				
One human	+	–	–	–
Six mouse	+	–	+/–	–
Mature T neoplasms				
One human	–	–	+	+
Two mouse	–	–	+	+

analysis, we have identified three different B-cell transforming genes and two different T-lineage transforming genes. In these groups, a common gene appears to be activated in both mouse and human neoplasms within a differentiated cell type, again suggesting the presence of evolutionarily well-conserved genes. The restriction analysis of each of these genes establishes them as distinct from transforming genes of human bladder carcinoma [6], mouse and human mammary tumors [8], and chemically transformed fibroblasts [4]. The transforming genes thus far identified correlate well with phenotypic expression within particular differentiated cell types. It is our hypothesis that these genes represent cell growth genes which have become freed of normal regulatory constraints. When cloning of a representative gene from each of the five groups is completed, we will use these genes as probes to determine what signals trigger their expression during normal cellular differentiation, and we hope to determine at which stage these genes are at risk of neoplastic changes.

Acknowledgments

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The Human *onc* Gene *c-myc*: Structure, Expression, and Amplification in the Human Promyelocytic Leukemia Cell Line HL-60*

R. Dalla Favera, E. Westin, E. P. Gelmann, S. Martinotti, M. Bregni, F. Wong-Staal, and R. C. Gallo

Substantial evidence indicates that retroviral transforming (*v-onc*) genes originated by means of recombination between a present nontransforming virus and normal cellular sequences (Duesberg et al., this volume). These sequences, called cellular *onc* gene, are highly conserved during evolution, suggesting that they may code for protein products which are essential for cell growth or tissue differentiation. As these normal cellular genes are homologous to viral- transforming genes, their potential role in tumorigenesis is of great interest. As an alternative to direct transformation by a viral *onc* gene, abnormal activation of a cellular *onc* gene may cause transformation. Two models have been proposed for such a mechanism. First, high levels of expression of a cellular *onc* gene may be caused by the insertion nearby of a viral promoter [12, 15, 16, 17] or by alteration of the physiological promoter by a mutagenic agent such as a chemical carcinogen. Secondly, a cellular *onc* gene may be relocated in a transcriptionally active region of the genome as a consequence of chromosomal rearrangements [2, 5, 6,13]. In this chapter we review the evidence for a possible third mechanism for *onc* gene activation in neoplastic cells, that of gene amplification. The human homologue, *c-myc*, of the transforming gene of avian myelocytomatosis virus (MC29), which is expressed at relatively high levels in the human promyelocytic leukemia cell line HL-60, is stably amplified in the genome of these cells [7]. Amplification was also detected in primary, uncultured leukemic cells from the same individual, suggesting that the *c-myc* amplification may have been involved in the leukemic transformation in this case.

A. Genomic Organization of Human *c-myc* Sequences

The avian myelocytomatosis virus genome (MC29) contains an *onc* gene, *v-myc*, coding for a DNA-binding nuclear protein which is responsible for the transforming ability of the virus (Moelling et al., this volume) [9]. A recombinant plasmid (pMC0) containing the entire *v-myc* gene was derived from an integrated provirus clone (Papas et al., this volume) [14]. Hybridization of pMC0 to normal human genomic DNA indicated that multiple regions in the human genome contain sequences related to *v-myc* [8]. In order to establish the genomic organization of these sequences a human recombinant DNA library was screened using pMC0 as a probe, and five recombinant phages were isolated (λ-LMC-3-4-12-26-41) [8]. Restriction enzyme analysis of λ-LMC-12 and 41 determined that these two clones share approximately 17 kb DNA where restriction sites are conserved, suggesting that they represent the same genomic segment (Fig. 1). The 8.2-kb *Hind*III-*Eco*RI fragment was isolated by preparative electrophoresis and analyzed by hybridization of Southern blots to *v-myc* probes (Fig. 1 B). Hybridization of pMC0 to *Cla*I-*Sst*-I digests (Fig. 1 C) shows that

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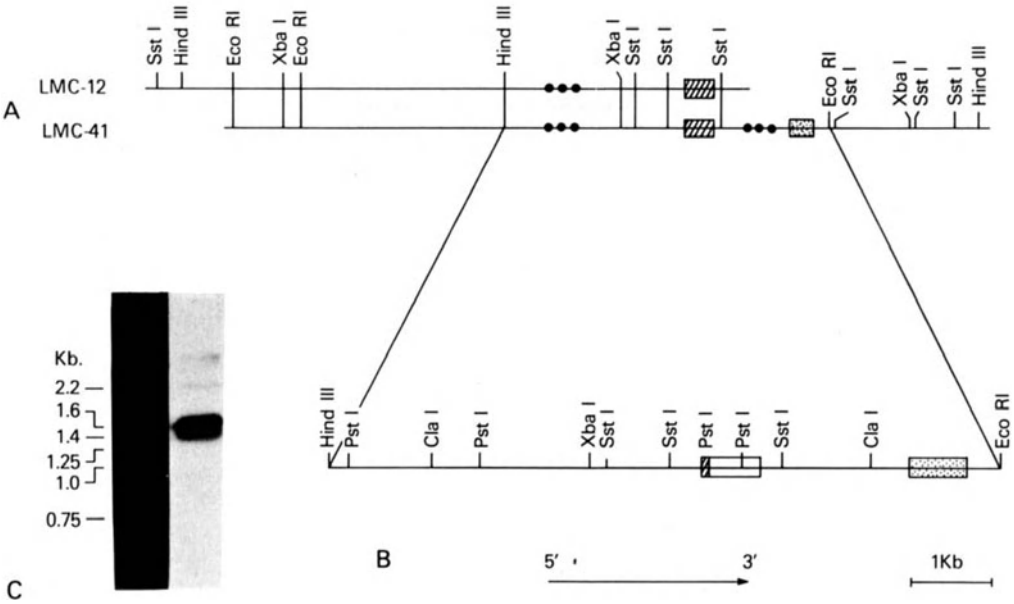

Fig. 1A–C. Genomic organization of the *c-myc* gene. **A** Restriction map of λ-LMC-41 and 12 DNA inserts. **B** Detailed restriction map of the 8.2-kb *Hind-Eco*RI fragment. *Cross-hatched box,* hybridizing to a 5′ probe fragment; *white box,* pMCO hybridizing fragments; *stippled box,* hybridizing fragment to a 3′ probe; ●●●, fragments containing *Alu* repets. **C** Right, ethidium bromide staining of DNA fragments generated by *Sst*I-*Cla*I cleavage of the fragment shown in **B** *Left,* hybridization to pMCO showing the two hybridizing fragments [8]

the region of hybridization is not continuous. A 1.0-kb *Cla*I-*Sst*-I fragment does not contain *c-myc* sequences. This fragment, which probably represents an intron in the *c-myc* gene, contains sequences related to the Alu family of repeats (data not shown).

In order to investigate further this structure, heteroduplex studies were performed. DNA from the recombinant λ-LMC phages were used to form heteroduplex molecules with phage DNA containing the MC29 provirus (Fig. 2). In agreement with the restriction enzyme data, *v-myc*-homologous sequences in λ-LMC-41 are interrupted by a nonhybridizing segment. Heteroduplex measurements of the *v-myc* hybridizing region of the λ-LMC-41 insert match the length of the viral *onc* gene, 1.56 kb (T. S. Papas, personal communication). These data suggest that the 19-kb region of clone λ-LMC-41 contains the active functional *c-myc* gene. Furthermore this structure is analogous to one of the single-copy chicken *c-myc* genes [18], some of whose restriction sites have been conserved in the human gene (T. S. Papas, personal communication).

Restriction maps of λ-LMC-3, 4, and 26 DNA (Fig. 3) show that these clones represent nonoverlapping, although possibly contiguous, regions of the human genome. Unlike the hybridization analysis of λ-LMC-41, we were unable to detect nonhybridizing intervening regions in λ-LMC-3, 4, or 26. Heteroduplex formation with λ-MC29 DNA showed for these single DNA clones an uninterrupted region of homology ranging from 0.2 to 0.4 kb (Fig. 2). As seen in Fig. 3, these sequences are homologous to the central portion of *v-myc,* lacking 5′ and 3′ homologous sequences. Moreover, these incomplete sequences are more divergent from the viral sequences than the complete gene since stringent washing conditions abolished the hybridization with λ-LMC-3, 4, and 26 without diminishing the intensity of the signal in λ-LMC-12 and 41. These sequences may represent parts of different functional genes which are partially homologous with the *c-myc* gene. However, as described below, only one species of mRNA has been detected in several different human tissues tested [10, 20], and higher levels of his

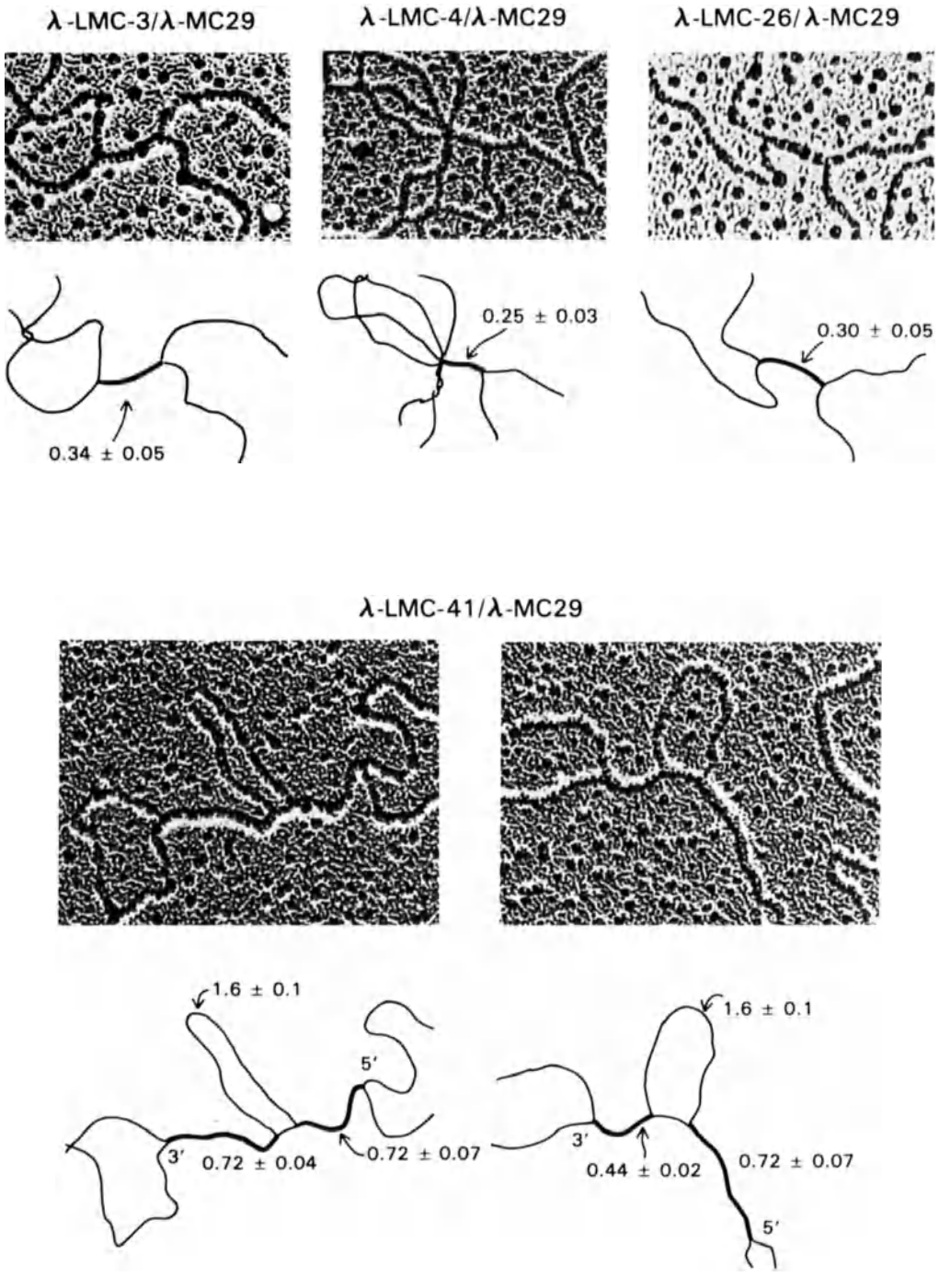

Fig. 2. Heteroduplex analysis of different human *c-myc* clones. The *top three panels* show the regions of the heteroduplex molecules containing the pseudogene hybridization. The *lower two panels* have the two kinds of molecules seen in the λ-LMC-41/λ-MC29 heteroduplex. Measurements are in kilobases [8] × 155,000

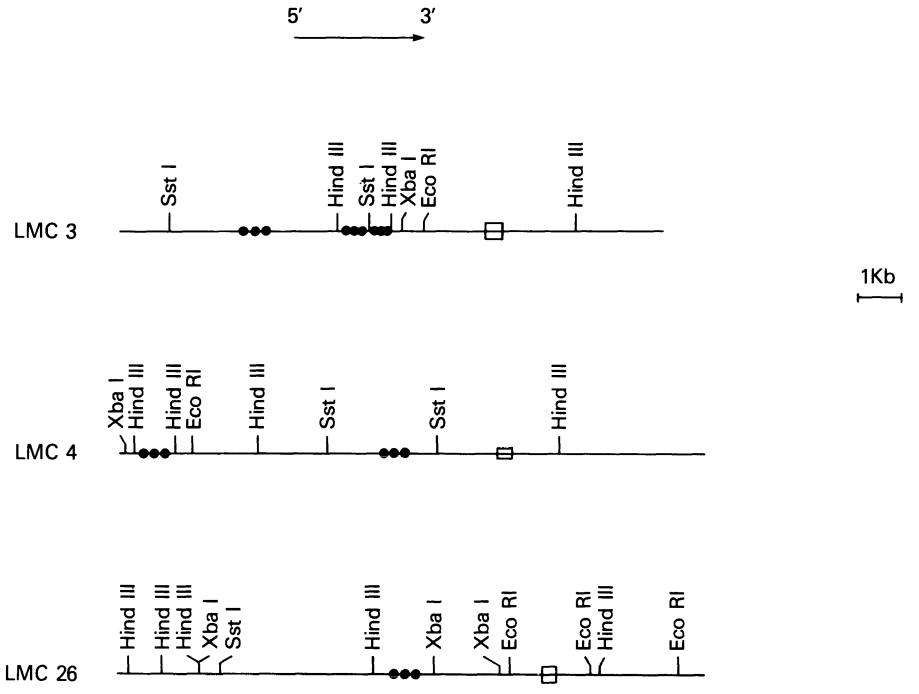

Fig. 3. Restriction maps of clones containing incomplete *c-myc* sequences. Symbols are as in Fig. 4 [8]

mRNA correlate with the amplification of the *c-myc* locus as cloned in λ-LMC-41 [7]. These data suggest that the additional, incomplete *c-myc* sequences may not be functional and may represent pseudogenes.

B. Expression of the *c-myc* Gene in Human Hematopoietic Cells

As detailed elsewhere in this volume (Wong-Staal et al., this volume) [20] the *myc* gene is transcribed into a single 2.7 kb mRNA transcript in all hematopoietic cells examined, where less then a two- to three-fold variation in the mRNA levels was detected. A single exception is HL-60, a human promyelocytic leukemic cell line [3], which expressed *myc*-related sequences at approximately a ten fold greater level compared with other cell lines. Because HL-60 has the unique capacity to differentiate into more mature myeloid cells after induction with several chemical agents, most notably Me_2SO and retinoic acid [1], we wished to determine whether expression of cellular *onc* genes is modulated as a function of myeloid cell differentiation. Figure 4A shows hybridization of the *myc* probe to RNA from untreated HL-60 cells and HL-60 treated with Me_2SO or retinoic acid. Expression of *c-myc* was reduced 80%–90% in HL-60 cells after induction with either Me_2So or retinoic acid. It seems likely that this gene was not expressed at all in the differentiated cell and that the residual band is due to the small population of undifferentiated cells hat persist in culture.

In contrast to *c-myc*, the *c-abl* gene remained expressed at similar levels in the differentiated and undifferentiated cells (Fig. 4B). These results also mitigate against the possibility that the apparent decrease in expression of *c-myc* is due to generalized RNA degradation in the mature granulocytic cells.

C. *C-myc* Amplification in HL-60 DNA

To investigate whether any structural alterations of the *c-myc* gene or adjacent regulatory regions could account for increased levels of *c-myc* expression which were present in HL-60, we analyzed the

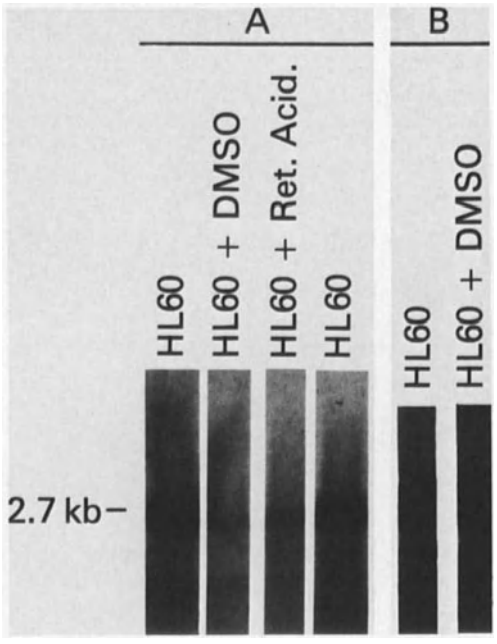

Fig. 4A, B. Hybridization of *myc* and *abl* probes to RNA from HL-60 induced to differentiate with various agents. Degree of differentiation was judged by the percentage of cells able to reduce the dye nitroblue tetrazolium (NBT), which is a histochemical marker of more mature myeloid cells, and by light microscopic examination of Giemsa-stined cells. **A** Hybridization of *myc* probe to two independent isolates of RNA. Lanes: *a* and *d,* from uninduced HL-60 (<2% of cells were NBT positive); *b,* RNA from HL-60 induced to differentiate with Me_2SO (87% NBT positive); *c,* RNA from HL-60 cells differentiated to 40% NBT-positive cells by using retinoic acid. **B** Hybridization of *able* probe to HL-60 (<2% NBT positive) RNA (lane a) and to RNA from HL-60 induced to 87% NBT positive with Me_2SO (lane b) [20]

structure of *c-myc* sequences in HL-60 by Southern blot hybridization. *Eco*RI digests of DNA from primary and cultured HL-60 cells, as well as from other normal and leukemic DNAs, were hybridized to a probe (pMCO) which contains the entire *v-myc* gene. This probe detects all *c-myc*-related sequences in the human genome, including the pseudogenes [8]. Fig. 5 shows that the 12.8-kb band, corresponding to the functional gene, was markedly increased in the two HL-60 samples, confirming the results shown in Fig. 1. One of the two DNA samples (Fig. 5) was isolated from primary uncultured HL-60 cells obtained from the patient's peripheral blood. This result proves that the amplification event was not a result of cell culture. In contrast, the intensity of the 5.8-kb band, which corresponds to one of the *c-myc* pseudogenes, is uniform in all the samples, suggesting that just the functional gene, but not the pseudogenes, is amplified. Moreover, this band serves a an internal control in proving that the difference in intensity of the 12.8-kb band is not due to experimental artifacts such as irregular DNA transfer or nonuniform hybridization across the nitrocellulose filter. In addition, a 4.6-kb fragment appears in the two HL-60 DNA samples which is not visible in the other samples. The origin of this fragment cannot be explained at the present time. We suggest that it could represent either an unidentified pseudogene which would be part of the amplification unit or a new fragment generated during the *c-myc* amplification event in HL-60 cells. Finally experiments were also performed to quantitate the amplification of *c-myc* in HL-60. For this purpose 30 μg *Sst*I digested HL-60 DNA was sequentially diluted as indicated in Fig. 6, and the intensity of the hybridization band was compared with the one obtained with 30 μg normal human lymphocyte DNA. Hybridization to a probe derived from another human *onc* gene, *c-sis,* which is a single copy gene [4], was used as control in the same experiment. Whereas the intensity of the *c-sis* band is comparable in normal spleen and undiluted HL-60 DNA, a 16- to 32-fold dilution is necessary to bring the *c-myc* band to the same levels as the control DNA. These data indicate that the *c-myc* gene is amplified between 16- and 32-fold in the HL-60 genome.

D. Summary and Conclusions

We have studied the genomic organization of *c-myc* sequences in the human genome. Analysis of different recombinat clones suggest the presence of at least one complete gene and several related sequences which may represent either distantly related genes or pseudogenes. Transcripts from the *c-myc* gene are detectable in a variety of human hematopoietic and non-

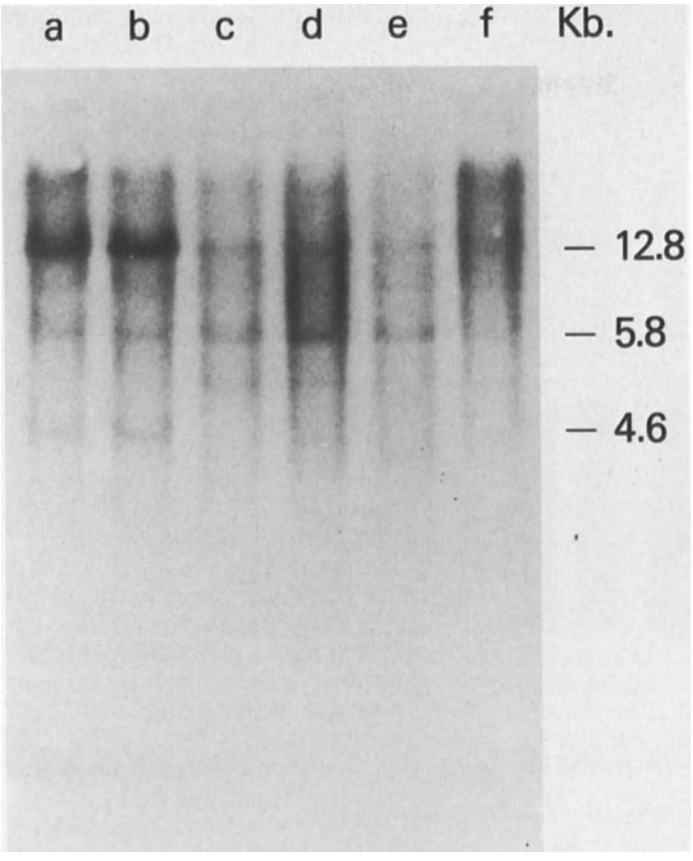

Fig. 5. Hybridization of HL-60 and other human DNAs to *v-myc* probe (pMCO). Thirty micrograms of DNA from different normal and leukemic cells were digested with *Eco*RI and hybridized to a *v-myc* probe (pMCO): *as,* HL-60 p. 70; *b,* uncultured leukemic HL-60; *c,* normal human spleen; *d,* peripheral blood AML cells; *e,* Molt 4 cell line; *f,* peripheral blood normal human lymphocytes [7]

hematopoietic cells, and increased levels of *myc* messenger RNA have been occasionally detected in some neoplastic tissues. The highest levels have been detected in the cell line HL-60 derived from neoplastic cells from a patient with acute promyelocytic leukemia.

Our data indicate that the *c-myc onc* gene is amplified in the genome of the HL-60 cell line, as well as in the original uncultured leukemic leukocytes which were obtained from the peripheral blood of the patient prior to any chemotherapeutic treatment [11]. The levels of *c-myc* amplification do not seem to vary during prolonged cell culture or after indicution to differentiation when the *c-myc* gene is no longer expressed. This result cannot be simply explained by amplification of specific chromosome(s) in these cells since they are hypodiploid, and no hyperdiploidy of individual chromosomes was present [11]. Since tissues other than the leukemic cells are not available from the patient we cannot determine whether *c-myc* was amplified in the other nonleukemic cells of the same individual. The amplification event may have occurred at the germ line level or may represent a normal event during myeloid differentiation analogous to the developmental amplifications of chorion genes during oogenesis in *Drosophila,* or actine genes during myogenesis in chickens (for review see Schimke [19]). Alternatively if *c-myc* amplification occurred just in the leukemic clone, it may

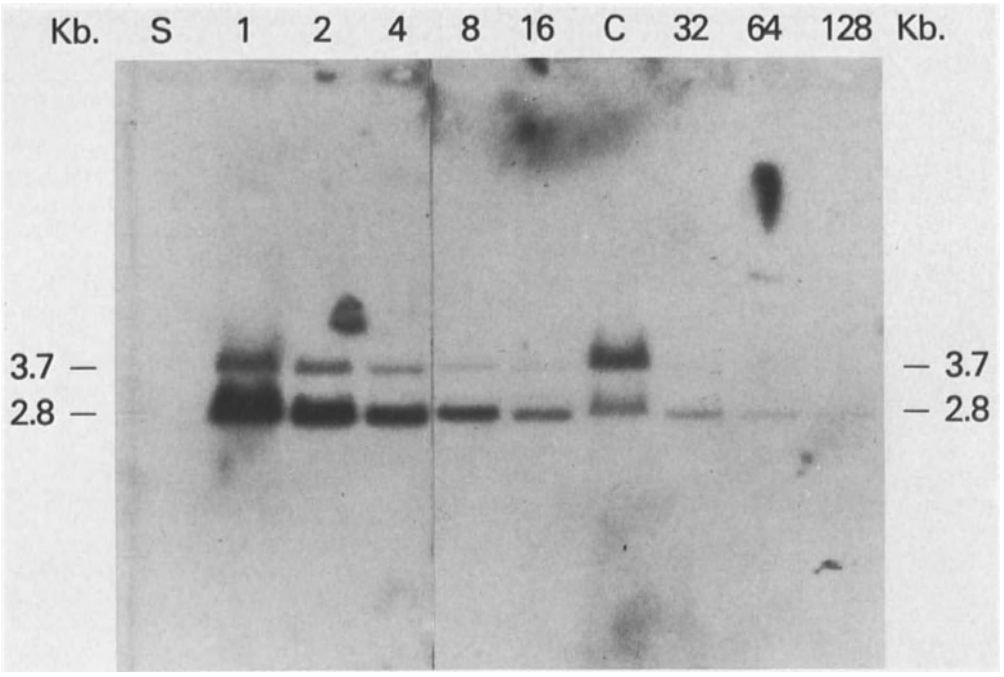

Fig. 6. Estimate of *c-myc* copy number in HL-60 DNA. For this Southern blot diution experiment all DNAs, as listed below, were digested with *Sst*I and simultaneously hybridized to pMC41-3RC and to a human *c-sis* probe (pL335). *C-myc* and *c-sis* hybridization bands in 30 μg DNA from normal human lymphocytes (lane *C*) were used as standards and their intensity compared with that obtained with the same amount of DNA from uncultured HL-60 cells (lane *1*) and serial dilutions of the latter with equal amounts of *Sst*I-digested salmon sperm DNA. The reciprocal of the dilution factor is shown above the lanes. Salmon sperm DNA was chosen as diluting DNA since *c-myc or c-sis* related sequences are not detectable under these conditions of hyridization, as shown in lane *S* (30 μg). The 2.8-kb *c-myc* band in lane *C* is intermediate in intensity between the bands in lane *16* and *32*. The 3.7-kb *c-sis* band closely matches the one in undiluted HL-60 DNA. Therefore, the *c-myc* copy number is between 16- and 32-fold higher in HL-60 compared with the normal standard. In contrast, the unamplified *c-sis* gene is present as a single copy in both genomes [7]

have been involved in the pathogenesis of leukemia in this case. However, the *c-myc* gene is not amplified in a few other cases of acute promyelocytic leukemia tested, suggesting that different mechanisms, including activation of different cellular *onc* genes, may lead to transformation even within similar neoplastic diseases. The validity of the *onc* gene amplification model can be tested in several different tumors expressing high levels of any of the known cellular *onc* genes.

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Determination of Virulence Properties of Leukemia Viruses *

W. A. Haseltine, J. Lenz, and R. Crowther

Understanding the basis of virulence of leukemia viruses is a major unsolved problem. Under natural and experimental conditions, retroviruses may replicate freely in the host with no profound consequence. Such is typically the case for vertically transmitted viruses that express early in life, e.g., the endogeneous baboon and murine Akv virus [1]. We call such viruses avirulent. Horizontally transmitted retroviruses usually induce disease, but typically only after a lengthy latent period and then in only a small fraction of infected individuals [2]. Viruses of moderate virulence include feline, bovine, and gibbon ape leukemia viruses. In these cases, rampant viremia is usually followed by immune suppression of the infection. Onset of frank leukemia is delayed by months or years and is a low probability event. The virus-associated with adult T-cell lymphoma in humans, HTLV, probably falls into this class of virus (Gallo et al., this volume). Some experimentally manipulated leukemia viruses are highly virulent. For example, the Moloney strain of murine leukemia virus induces a rapid (3 months to death) disease in a high proportion of injected animals.

To approach the problem of a murine leukemia virus, we selected avirulent and highly virulent strains for intensive study. Our initial goal was to isolate strains of murine leukemia virus that were isogenic except for the virulence phenotype. This criterion was met by isoloation of the SL3-3 virus from a lymphoid cell line derived from a thymus of a diseased AKR mouse. This virus induces terminal thymic leukemia in close to 100% of injected newborns of the low-incidence leukemia strain, C3H/f, NSF/N, CBA, and SJL, within 60–100 days. the Akv virus is avirulent in similar tests. The biological properties of this virus resemble closely those of the avirulent virus Akv. The viruses are N-tropic, ecotropic strains that form XC plaques. Both grow to similar titers on NIH 3T3 fibroblast cultures, and replicate to similar titers upon injection of newborn mice.

Analysis of the genomic RNA of Akv and SL3-3 viruses shows them to be closely related [3]. Oligonucleotide maps of several AKR viruses are presented in Fig. 1. Using this method, we estimate that the SL3-3 and the Akv virus differ by no more than 50–70 point mutations.

To further characterize the structure of these viruses, we obtained an infectious clone of the provirus of the SL3-3 strain. Comparative studies of the proviruses were done using an infectious clone of the Akv provirus provided to us by Doug Lowy [4]. Heteroduplex and restriction enzyme analyses confirm the close structural relationship between these strains [5]. Viruses produced by transfection of NIH 3T3 cells with these proviral DNAs have the same biological and virulence properties as the parental strains [5].

Our present strategy for further study of these involves:

Determination of the entire nucleotide sequence of both proviral DNAs. This information will provide definitive in-

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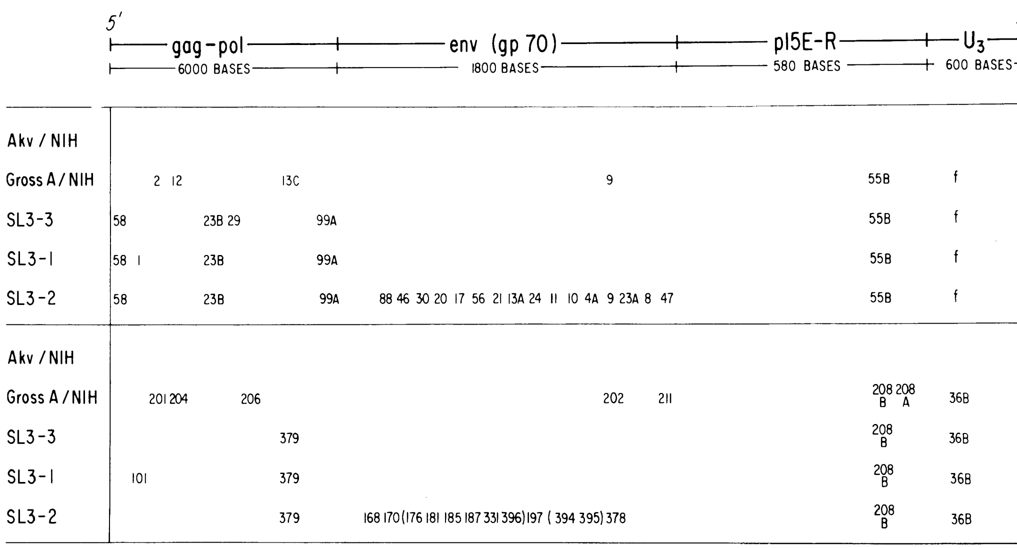

Fig. 1. Oligonucleotide maps of the AKR viruses. The *upper panel* represents oligonucleotides present in Akv which are missing from other viruses. The *lower panel* represents the positions of oligonucleotides which are not present in Akv. For the Akv genome, there are a total of 34 oligonucleotides within the *gag-pol* region, 16 within gp70, 9 within p15E-R, and 6 within U_3

formation regarding the structural differences in the viral genomes.

Construction of recombinant viruses, using proviral DNA intermediates, to localize virulence determinants.

Progress to date includes:

1. Determination of the sequence of the gp70-p15E region encoded by the Akv virus [6]. Analysis of this sequence permits construction of a speculative model for association of this protein with the viral membrane (Fig. 2A, B). Comparison of the sequence of the Akv virus with that of the SL3-3 virus and other vertically transmitted and other virulent strains isolated from AKR thymoma cell lines show a common divergence in the p15E region. The arg-leu to his-met change indicated in Fig. 2B occurs at a site at which the postulated "anchor" of the p15E protein enters the membrane. This change may have a physiological consequence.

Another change in sequence of the virulent versus avirulent strains was detected in the noncoding LTR region of the virus. A single point mutation from A to G located near the 5′ end of the LTR (58 to f, Fig. 1) is characteristic of the virulent strains.

2. Construction of a virulent recombinant virus. A recombinant virus produced by reassortment of fragments of the Akv and the SL3-3 proviruses was used to infect newborn mice. The structure of this recombinant is pictured in Fig. 3. The virus induced thymic leukemia in most of the injected animals. The latent period of disease onset was similar to that of the SL3-3 strain itself. In this recombinant, most of the LTR (all but the 5′ 36 base pairs), the entire *gag* region as well as half of the *pol* region of this virus is of SL3-3 origin. The 3′ half of the *pol* gene and the entire gp70-p15E, as well as the noncoding region including 36 bases of the 5′ end of the LTR, is of Akv origin.

The simplest explanation for these data is that changes in the gp70-p15E region do not effect the virulent phenotype, nor do changes in the 3′ region of *pol.* Changes in the LTR, *gag,* and 5′ region of *pol* are, therefore, implicated in the virulent phenotype. However, a firm conclusion is somewhat premature as the properties of the reciprocal recombinant are still not known. Nonetheless, the results of such recombinant constructions should permit

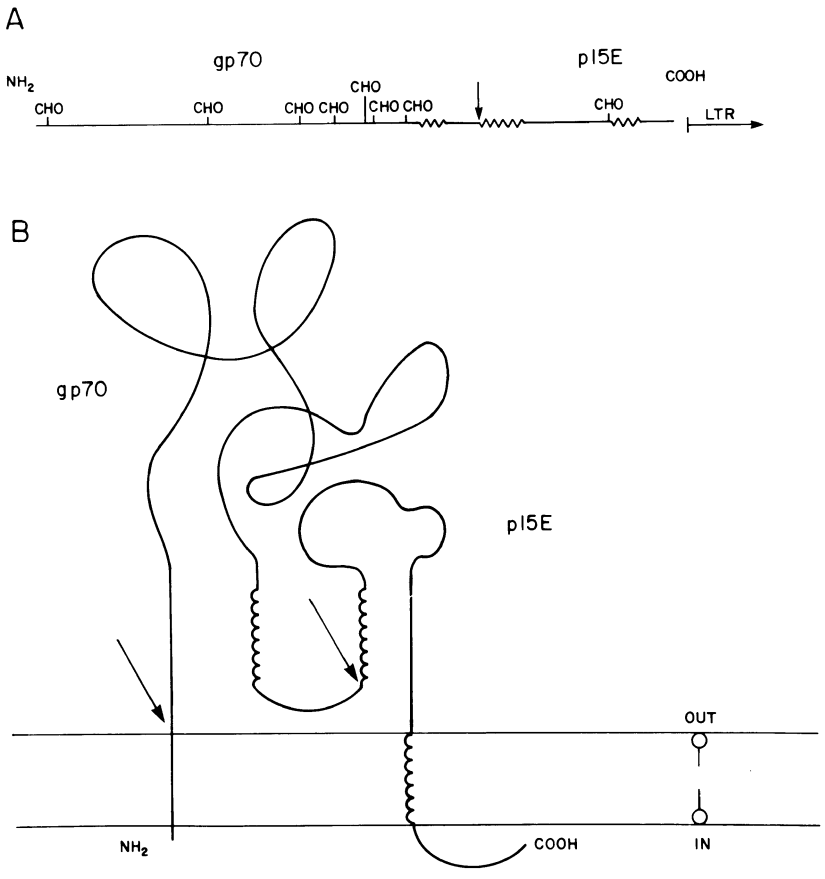

Fig. 2 A, B. **A** Linear representation of the *env* precursor from the amino terminus of gp70 ot the carboxyl terminus of the precursor. The amino terminus of pl5E is indicated by an *arrow,* as is the position of the boundary of the LTR. Potential glycosylation sites (Asn-X-Thr or Ser) are indicated by CHO. Regions of uncharged, hydrophobic amino acids are indicated by *wavy lines.* Three of these regions are present, one near the carboxyl terminus of pl5E. **B** Figurative representation of the potential structure of gp70 and pl5E relative to the lipid portion of the membrane. Four potential membrane traversal regions exist. These are the leader signal sequence at the amino terminus and the three regions described above. The latter three are depicted by *short loops.* According to this structure, only the hydrophobic region near the carboxyl terminus of pl5E is located in the lipid bilayer in mature peptides. The relatively hydrophilic regions which form most of the peptides are indicated by the *curved lines.* The *arrows* indicate the amino terminus of gp70 after removal of the leader sequence and the amino terminus of pl5E. No implication of secondary or higher order structure is intended, except that the three potential membrane traversal regions are sufficiently long to be capable of crossing the membrane in a helical configuration

definitive localization of virulence determinants.

Structural analysis of genomic determinants of virulence will be of value, provided they shed some light on the biological aspects of viral induction of leukemia. In this regard, the multistep nature of the leukemia process must be remembered. Probable steps include:

Initial infection of the animal
Viremia
Immune response to viremia
Infection of critical target issues
Preleukemia (proliferation of target tissues)
Frank leukemia (fixation of the malignant tumor cell)

The Akv and SL3-3 viruses seem equally proficent with regard to the first two steps.

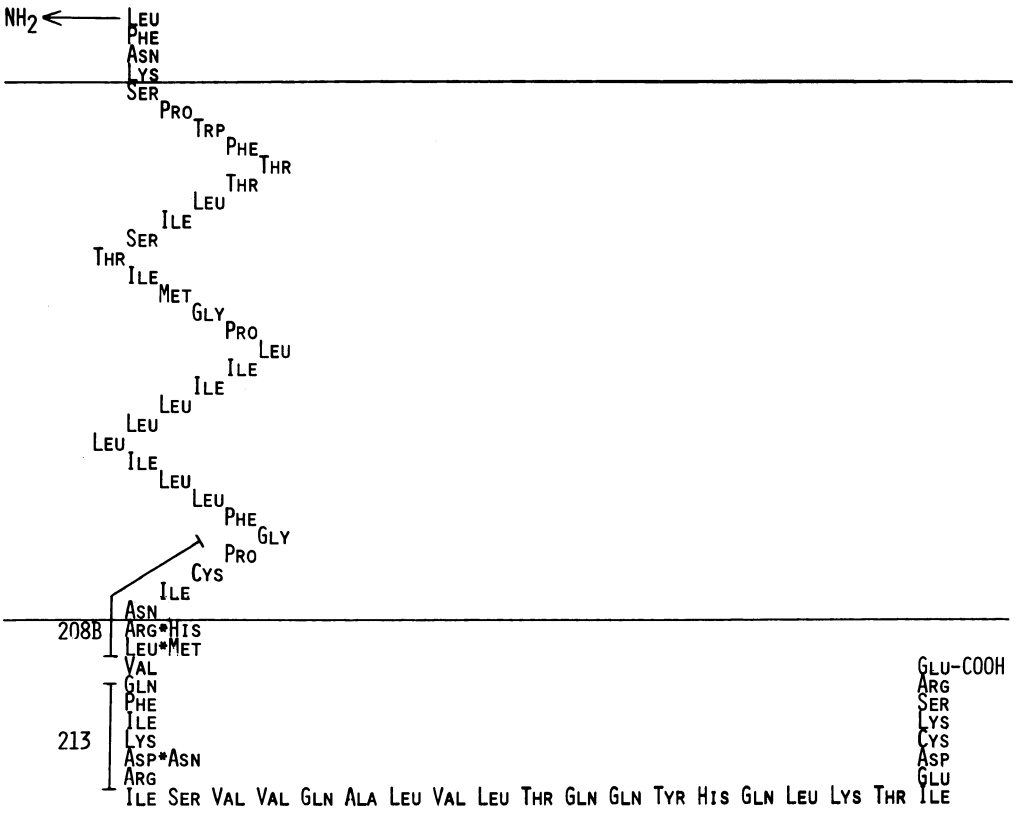

Fig. 2C. Localization of changes in the ecotropic, leukemogenic virus relative to the Akv genome in the anchor region of the pl5E proteins. The Akv sequences presented show the membrane traversal region near the carboxyl end of pl5E. The positions and amino acid changes of the Gross A/NIH oligonucleotides relative to Akv are shown. Oligonucleotide 208 b is shared by Gross A/NIH, SL3-3, and SL3-3. This oligonucleotide is also found in viruses produced by a number of other thymoma cell lines established from spontaneous tumors of AKR mice

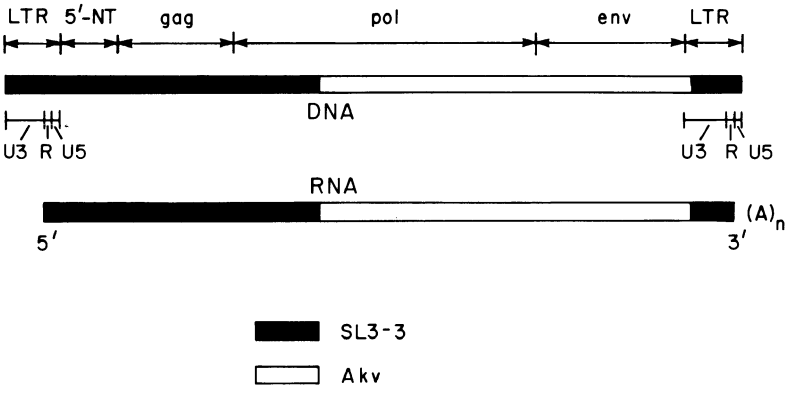

Fig. 3. Construction of a recombinant DNA provirus from infectious Akv and SL3-3 proviral DNAs. The recombinant provirus was constructed and the location of the SL3-3 and Akv components verified by restriction enzyme mapping. DNA from this virus was used to transfect NIH 3T3 cells so as to produce infectious virus. This virus accelerates the onset of disease in AKR mice, with a latent period similar to that of the SL3-3 virus. The virus also induces disease in CBA mice with a short latent period at high incidence

The immune response to the viruses is as yet incompletely characterized. There may be important differences in infection of the target tissue. Preliminary evidence suggests that even though both viruses may infect T-cell cultures in vitro the virulent strain replicates much more efficiently in such cells than does the avirulent Akv virus (Haseltine, unpublished results). Restriction of replication in the target tissue might be a sufficient explanation of the virulent phenotype.

Events that trigger cell proliferation in the preleukemic phase of the disease are poorly characterized. Typically, preleukemic cells are not transplantable and do not establish themselves in cell cultures. Evidence from the Friend leukemia system suggests that the envelope glycoprotein determinants may play a direct role in preleukemic proliferation [7] – a proliferation of lethal consequence in the case of Friend virus. Subtle alterations in *env* gene structure may affect this phase of disease for the AKR viruses as well.

There is mounting evidence that the long latent period and low disease incidence of virally induced leukemias can be attributed to the stochastic nature of events that occur subsequent to infection of the target cell population. Such events may include activation of cellular genes such as *myc, erb,* and *sis* by insertion of proviral DNA in nearby cellular sequences [8]. Chromosomal rearrangements or duplications (for example, trisomy 15 in the case of murine T-cell leukemias) may also represent late events in this process [9]. Changes that do not involve either mutations or genetic rearrangements in cellular DNA, but do affect, in a metastable way, the cellular program, may also be involved in fixation of the malignant cell phenotype. A sequence of such events might also be necessary before a fully malignant phenotype develops. It does seem likely that a precondition for the late events is creation of preleukemic proliferative population within the target cell pool. Such a population of preleukemic cells may result either from viral infection, or possibly from chemical exposure. The role of the virus in the later stages of the disease has yet to be fully established.

The murine system has several advantages for approaching these problems. A series of well-defined, isogenic viral reagents can be constructed, and the consequence of each change determined. Moreover, the entire process, from infection to frank leukemia, occurs within a 2-month period, in almost 100% of the injected animals. It is our hope that insights obtained in this system will be applicable to understanding the disease process in such cases as the adult T-cell leukemia of humans that arise under natural circumstances.

Note in Added Proof

Complete leukemogenicity data is now available for the recombinant genome shown in Fig. 3. Viruses with this genomic structure induced disease in 10 of 11 AKR mice and 3 of 10 CBA mice, showing that the SL3-3 segment of the recombinant genome contains the virulence determinants. Viruses were also derived by transfection of recombinant genomes with the reciprocal structure of the genome shown in Fig. 3. Viruses of the opposite structure were nonleukemogenic (0 of 22 AKR mice, and 0 of 10 CBA mice). Since these viruses contained the 3′ half of the SL3-3 *pol* gene and the entire SL3-3 *env* gene, this result clearly demonstrates that this segment of the genome does not encode the virulence determinants.

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Modern Trends in Human Leukemia V
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Generation of Recombinant Murine Leukemia Viruses De Novo: An Alternative Model for Leukemogenesis

P. J. Fischinger, N. M. Dunlop, C. M. Poore, and W. G. Robey

A. Summary

Evidence for the viral etiology of murine lymphoma is very complex. Although ecotropic viruses were considered in the past to be the causative agents, envelope gene recombinant (RM) types of murine leukemia virus (MuLV) seem to be the best candidates at present. The following factors are relevant in the murine model: Pure ecotropic MuLV causes disease but induces RM-MuLV de novo in every case. RM-MuLV can cause disease in pure form. However, the mere presence of RM-MuLV may not be sufficient to cause disease in some cases. Ecotropic MuLV is needed under natural conditions for inducing T-cell blastogenesis, donating its coat to recombinant MuLV, and to become a partial parent for RM-MuLV. Early elimination of ecotropic MuLV can prevent disease. In a number of virus-free mouse lymphomas, the only indicator of virus involvement was on RM-MuLV glycoprotein on cell surface. Based on the above, a signal hypothesis model is proposed which attempts to integrate the above observations.

B. Introduction

Murine lymphoma has been one of the earliest and most explored models in both viral and physical carcinogenesis. Despite enormous amounts of data on the role of many classes of viruses, genes, and genetically pure mouse strains, no clear-cut views of a common mechanism of etiology had been formulated. At present, there are several candidate classes of murine leukemia viruses (MuLV) which could be involved in causality. These include ecotropic (E) agents which grow only in mouse or rat cells, xenotropic agents (X) which are endogenous to mouse DNA but grow only in nonmouse cells, and viruses which have a polytropic host range. The last group includes wild mouse amphotropic C-type viruses and viruses which appear to be envelope (*env*) gene recombinants (RM) of E and X nucleotide sequences. The *env* gene product of RM-MuLV is a permuted glycoprotein, gp70, molecule identifiable by various biological and physical parameters. It is only the latter type of viruses which seems to be critically involved in leukemogenesis [6, 14]. At least some RM-MuLV could induce leukemia and fulfill the postulates of causality [7]. On the other hand, the role of these viruses in disease is very complex, and generally they are not detectable in E-MuLV stocks because they are masked with the ecotropic *env* gp70 coat [8]. However, a very simple question had not been answered: Could pure E-MuLV cause leukemia by itself or is generation of RM-MuLV required in every case? The data below unambiguously answer this question and are fitted into an alternative model for leukemogenesis.

C. Composition of MuLV Stocks

When standard leukemogenic strains of MuLV are tested for the presence of RM-MuLV or X-MuLV, they are almost always negative. However, when tested for the genomic content of individual infectious units, stocks of Moloney (M), Friend, Graf-

Procedure (Time and number of tests)	Virus assays FIU/ml in [a]	
	Mouse indicator cells	Cat indicator cells
A. Direct assays of virus from SC-1 cells (3 yrs, ten tests)	5×10^6	0
B. Passage of virus through virus-free thymoma cells – Swiss and C57BL (2 yrs, 12 tests)	4×10^4	0
C. Passage of virus through mouse S+L– cells: by assay of pseudotype host range (2 yrs, six tests)	7×10^5	0
D. Assay of property of individual infectious units (65 tested)	65/65 positive	0/65 positive

[a] Indicator cells were S+L– FG/10 mouse or clone 81 cat cells for leukemia viruses and normal mouse 3T3FL cells and normal feline fibroblast cells for the assay of MSV pseudotype viruses in C

Table 1. Genetic purity and stability of ecotropic Moloney MuLV passed in tissue culture

fi, and Gross MuLVs all contained RM-MuLV between 1%–10% of total viruses [10]. The strain 1869 of M-MuLV was cloned by single focus isolation and end-point limiting dilution techniques (M-1869). The genetic purity and the ecotropic stability of this virus were ascertained by various procedures. As seen in Table 1, this virus grew to high titers in normal mouse fibroblast cells, and in 3 years of passage never generated any virus with an extended host range on direct testing in mink or cat sarcoma-positive, leukemia-negative (S+L–)cells. Virus was also passed through several types of virus-free mouse thymic lymphoma cells in culture. In several years of testing in E-MuLV nucleotide sequence- free Swiss lymphoma cells (NIXT line) or in the C57B1 RL-12 lymphoma line, no recombinant virus was generated. Extensive unmasking experiments were performed by checking whether RM-MuLV was contained in an ecotropic MuLV coat because of serum oncornavirus inactivation factor (OIF) selection pressure [21]. This was done most simply by passing cloned M-1869 through mouse S+L– cells and checking whether any MSV progeny virus was detected which could form foci in cat or mink cells [7]. No RM-MuLV was detected at a sensitivity level of $\leqq 10^{-6}$. Finally, individual infectious units of M-1869 were tested for their genomic content by infecting microtiter wells containing mouse S+L– cells at an effective e.o.p. of 0.2. The progeny of all 65 single infectious unit positive wells was strictly ecotropic MuLV, indicating that RM-MuLV was not induced in tissue culture and that mixed "diploid" genomes were not detected. Accordingly, this M-1869 stock was considered to be free of associated RM- or X-MuLVs.

D. De Novo Induction of Recombinant MuLV in Vivo

The cloned pure ecotropic M-1869 was inoculated into newborn Balb/c mice. After an inoculum of 2×10^5 FIU of M-1869 per mouse, the animals rapidly became viremic and leukemia developed in ~90% of the animals in 3–5 months. The disease was compatible with the previously described Moloney syndrome [5]. Table 2 presents data on virus status on 32 individually tested Balb/c mice in various stages of their disease. High titers of virus were seen in the plasma within a week after inoculation. The animals continued to be viremic, but no free or masked X- or RM-MuLV was detected until ~60 days of age, when about half of the animals had very low amounts

Table 2. Generation of RM-MuLV in vivo after ecotropic M-MuLV inoculation

Balb/c mice age in days (condition)	Mice[a] tested	MuLV titer (FIU/ml)			% RM-MuLV[b] of total virus (number of mice +)
		Direct in mouse S+L−	Direct in cat S+L−	Masked RM-MuLV	
7–30 (viremic)	14	$\geqq 10^6$	0	0	0 (0)
30–60 (viremic)	4	1.3×10^6	0	2.9×10^2	0.02% (2)
60–90 (preleukemic)	4	9.8×10^5	0	4.3×10^3	0.44% (4)
75–150 (leukemic)	10	9.4×10^5	0	2.7×10^4	2.9% (10)

[a] Pooled organ extracts from each mouse were tested individually. In some cases, the thymus, spleen, or liver were tested separately

[b] Average values of all individuals. In the leukemic population RM-MuLV as percent of total virus varied from 0.33% to 6.8%

of masked RM-MuLV in their plasma. During the preleukemic phase, all the animals had detectable masked RM-MuLV (0.5% of all ICCs). During the early frank leukemic phase, the E M-1869 virus titer remained 10^6 FIU/ml, but no free RM-MuLV was detected. These animals had a high level of circulating OIF in their plasma. However, about on average 2.9% of all virus was masked RM-MuLV. In *every* case of leukemia, new RM-MuLV was detected. The presence of this virus was coincidental with disease, and had to arise de novo.

To test these phenomena more precisely, infectious cell center (ICC) experiments were carried out. Lymphoid cells in thy-

Table 3. Infectious cell centers by lymphoid cells from organs of M-MuLV 1869 inoculated Balb/c mice

Days after infection	Organ	Percent of infectious cell centers[a]	
		Ecotropic: on mouse S+L− cells	Recombinant: on cat S+L− cells
10	Thymus	0.8	$<10^{-6}$[b]
	Spleen	5	$<10^{-6}$
20	Thymus	11	$<10^{-6}$
	Spleen	3	$<10^{-6}$
30	Thymus	22	$<10^{-6}$
	Spleen	9	$<10^{-6}$
40	Thymus	15	0.005
	Spleen	9	$<10^{-6}$
57	Thymus	21	0.3
	Spleen	7	$<10^{-6}$
150	Thymus	10	0.04
	Spleen	8	0.01

[a] From 10^3 to 10^6 twice-washed lymphoid cells from freshly harvested organs were added to mouse or cat S+L− cells. Foci of infections were counted 6 and 12 days later in mouse or cat S+L− cells respectively

[b] Lower level of sensitivity: no foci detected at 10^6 cells/dish

muses and spleens were tested by using dilutions of washed cells (10^6–10^3 per dish of mouse or cat S+L– cells) and determining the proportion of cells yielding virus in either test system. Table 3 shows virus growth in cells of thymuses or spleens. Very rapid growth of virus occurs during the first week of life in the cells of both organs. Between 10% and 25% of all lymphoid cells could act as ICCs for ecotropic MuLV, which is 10–1000 fold higher than in the endogenous AKR disease development [12]. The high ecotropic MuLV titers persist throughout life and into the leukemic phase. When the aliquots of the same cells were tested in cat S+L– cells to determine whether they produced RM- or X-MuLV, no RM-MuLV producing cells were apparent until about day 40. After that, essentially all animals were positive for RM-MuLV regardless of time of testing. Virus-positive cells were present essentially only in the cells of the thymus and not in the spleen or the liver. RM-MuLV positive cells were detected in other organs only during late stages of disease. The rather strict appearance of RM-MuLV only in the thymus underscores the fact that recombination generating RM-MuLV probably took place only in the thymic environment.

E. Properties of the Induced RM-MuLVs

It is generally known that individual RM-MuLVs, derived even from a single type of MuLV, exhibit a remarkable variety of changes in their *env* gene coded gp70 product [4, 13, 23]. Additionally, RM-MuLVs derived from Moloney stocks had Moloney-specific p12 and p15 peptides [9]. It was of interest to test the nature of newly derived RM-MuLVs from the relatively

Table 4. Envelope characteristics of selected RM-MuLVs derived from Moloney ecotropic MuLV

Property measured	Virus isolate					
	RM-M_{HIX}	RM-Mp_1	RM-M_{57}	RM-M_{PB9}	RM-AKR_{Tul}	*Eco*869
Growth in mouse S+L– cells	$\geqq 10^5$[a]	$\geqq 10^5$	10^2	10^3	$\geqq 10^4$	1×10^6
Growth in mouse fibroblasts (SC-1)	$\geqq 10^5$	$\geqq 10^5$	10^3	$\geqq 10^4$	NT	6×10^6
Growth in cat S+L– cells						
Type of focus	Syncytial	Syncytial	Syncytial	Syncytial	Round cells	0
In cat or mink cells	$\geqq 10^5$	$\geqq 10^5$	$\geqq 10^2$	$\geqq 10^4$	$\geqq 10^4$	0
Interference pattern[b]	RM-type	RM-type	RM-type	RM-type	RM-type	Ecotropic type
Neutralization with[c]						
Anti M gp70 MAb	+	+	+	+	–	+
Anti xenotropic gp70 serum	–	–	–	–	–	–
Anti AKR gp70	–	–	–	–	+	–
Sensitivity to OIF[d] (normal mouse serum)	+	+	+	+	+	–

[a] Focus-inducing units/ml; NT, not tested

[b] Interference patterns can be of three types: The ecotropic type tested by using free ecotropic F-MuLV (2 μg/dish) gp70 on mouse cells which reduces the number of ecotropic MuLV FIU by ≧90% [15]; The RM-type, in which a preinfection of either a mouse or nonmouse cell induces complete interference with any other RM-MuLV, the xenotropic type in which a preinfection of a nonmouse cell with xenotropic MuLV prevents growth of both RM-MuLV, and xenotropic MuLV. RM-AKR_{Tul} was isolated from the plasma of an uninoculated leukemic AKR mouse [10]

[c] Monoclonal antibody (MAb) 1D11 against ecotropic Moloney MuLV gp70 produced in tissue culture neutralizes only ecotropic Moloney MuLV but not Friend, Rauscher, Graffi, or AKR MuLVs [2]

[d] Normal STU mouse serum used at 1:100 dilution, which neutralized about 99% of virus

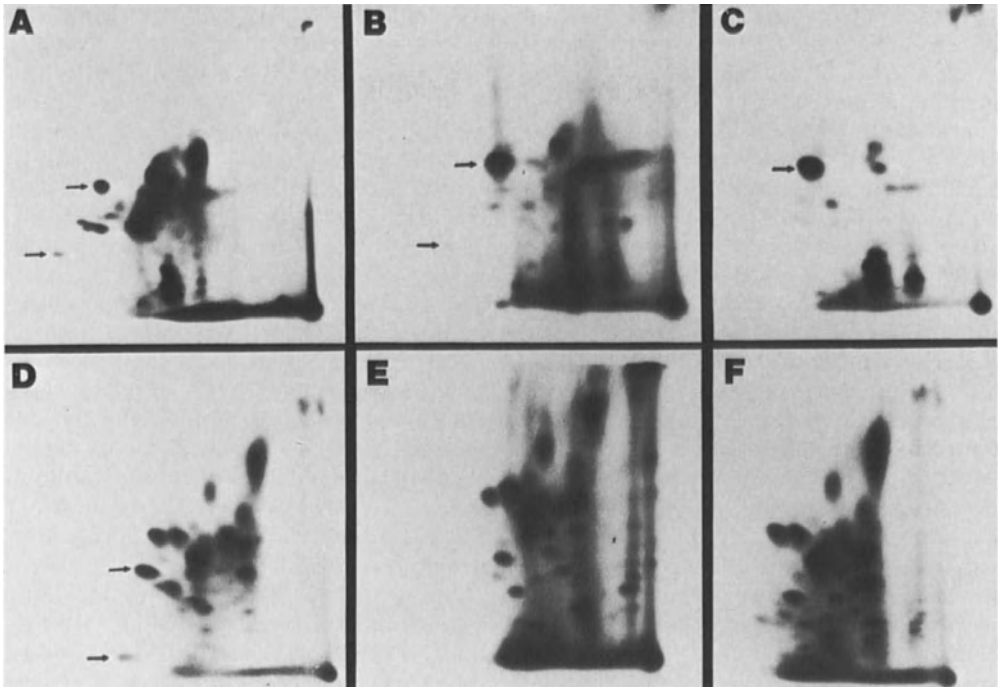

Fig. 1. Comparison of tryptic (*panels A–C*) and chymotryptic (*panels D–F*) peptides of gp70 polypeptides isolated from 1869-MuLV (*panels A, D*), RM_{57}-MuLV (*panels B, E*), RM_{PB9}-MuLV (*panels C, F*) cell culture fluids. The *arrows* denote specific peptides discussed in the text. The origin is at the *lower right* in each panel with the first dimension electrophoresis to the *left side* and second dimension ascending chromatography to the *top* in each panel. Technical aspects of iodination and two dimensional maps have been described [4]

early stages of the infective process. Accordingly, we isolated one RM-MuLV from a healthy, viremic mouse 56 days after inoculation (RM-M_{57}), and another from an early leukemic phase (RM-M_{PB9}). Two or more cycles of single focus isolation and limiting dilution isolation in cat diploid cells were performed in each case. What became apparent was that in terms of growth, RM-M_{57} did not grow avidly in any cell tested, but had a definite predisposition for growth in mouse cells (Table 4). In contrast, RM-M_{PB9} grew substantially better, yet favored heterologous cells in which it grew to higher titers. Both of these viruses are in contrast to other standard isolates such as RM-M_{HIX} or RM-M_{PI}, which grew equally well to higher titers in both mouse and nonmouse cells. However, all of the RM-M-MuLV derivatives produce very distinct syncytial foci in cat S+L– cells in contrast to RM-MuLVs from other types.

On testing of further *env* properties of these de novo isolated viruses, it was clear that all were very susceptible to mouse serum OIF as other RM or X-MuLVs. Neither virus was susceptible to any major degree to type-specific anti AKR or anti X-MuLV gp70 specific antisera. A clear relationship was detected to the presumed parental ecotropic M-MuLV gp70 in that specific neutralization was observed with a monoclonal antibody to Moloney gp70, which neutralized only M-MuLVs. Interference patterns of the new isolates are being examined whether they also follow a unique one-way interference pattern as described [9]. It is clear that although RM-MuLV isolates share an interference group, they are not interfered with by ecotropic gp70 and that X-MuLV can penetrate the RM-MuLV preinfection barrier in heterologous species cells [10].

The tryptic and chymotryptic oligopeptides of gp70 molecules isolated from M-

1869, RM-M_{57}, and RM-M_{PB9} are shown as two dimensional peptide chromatograms (Fig. 1). The tryptic map of M-1869 (panel A) shows greater peptide complexity than those of RM-M_{57} (panel B) and RM-M_{PB9} (panel C). However, similarities are apparent especially in the central region of the maps. Also, a higher degree of overall homology exists between RM-M_{57} and RM-M_{PB9} as contrasted to M-1869. A major peptide, denoted by the upper arrow in all three maps, has been described as a common marker for most but not all recombinant and xenotropic gp70s [4]. However, in this case, pure ecotropic M-1869 from SC-1 cells also displays a peptide in this region. A second minor peptide was also detected in M-1869 and RM-M_{57} that was not detected in RM-M_{PB9} gp70 (lower arrow). On the basis of the tryptic maps, it is clear that M-1869 has made a substantial contribution to the oligopeptide content of both recombinant viruses.

The chymotryptic maps for the same glycoproteins were also compared in Fig. 1. A striking degree of homology exists between the three glycoproteins, most noted between RM-M_{57} and RM-M_{PB9}. RM-M_{57} gp70 does contain a series of unique peptides of low electrophoretic mobility. The M-1869 contains two peptides unique to itself (arrows in panel D). The peptides in the lower right region of the maps of the presumed recombinant gp70s appear to contain the P32 group described as pathognomonic for RM-MuLVs [22].

F. Factors Required in Murine Viral Leukemogenesis

At this time, two other sets of phenomena besides the de novo induction of RM-MuLV seem to be operative in murine leukemogenesis which complicate the picture. The first factor is that the presence of an ecotropic virus during the first few days of life is mandatory. The best evidence for this is the fact that either in the spontaneous AKR or in the genetic M-MuLV disease, treatment with broadly neutralizing anti MuLV gp70 serum neutralized the ecotropic MuLV, and eliminated or reduced the ICCs, and prevented disease effectively [12, 24]. The second feature is that the mere presence of RM-MuLV may not be enough to generate disease. A salient example is the CBA/N mouse in which ecotropic M-MuLV could grow as well as in other mouse strains, yet which does not come down with leukemia [19]. The CBA/N mice do not respond in T-cell blastogenesis assays with ecotropic MuLV gp70s. This feature is under maternal genetic control and can be tested how leukemia incidence segregates in crosses. For example, in Balb/c×CBA/N F_1s (Balb/c mother), both males and females become both viremic and leukemic. However, in F_1s which have the CBA/N as mother, the females become leukemic, but the F_1 males are viremic but not leukemic. In collaboration with J. Lee and J. Ihle, we examined a number of sets of animals which were originally inoculated with a noncloned stock of M-MuLV. Specific testing for RM-MuLV as masked virus revealed that pure CBA/N mice which were viremic had both ecotropic M-MuLV and RM-MuLV. Similarly, in the F_1 cross with Balb/c mice, both variable sets and both sexes had similar amounts of masked RM-MuLV whether they developed leukemia or not. It is clear that at least in this genetic strain, RM-MuLV may be requisite, but of itself not sufficient for the development of leukemia. Because this strain has an inoperative system for blastogenesis after ecotropic MuLV gp70 stimulation, it may be inferred that an expanded lymphoid target cell population is also critical.

G. Role of RM-MuLV in Virus-Free Lymphomas

Despite the plethora of viral agents causing leukemia in the mouse, a classical phenomenon is the induction of a high percentage of leukemia in several strains of mice with X-rays [17]. Many of these leukemias are free of virus. Although in some cultured lymphoma lines, various MuLVs are released, many of these cultured lymphoma cell lines are free of detectable virus and viral antigens. A Swiss X-irradiation induced virus-free thymoma cell line (NIXT), and the RL-12 virus-free lymphoma were examined [11, 16]. Viral antigens were not detected with standard reagents, but in

each case antiserum against the RM-MuLV VL3 gp70 isolate stained a portion of cells in immunofluorescent assays [3]. The cells were highly resistant to infection with RM-MuLV, but not ecotropic MuLV. In both cases, a form of gp70 could be found on the cell surface and in the supernates. Based on peptide mapping, it was clear in both cases that there was a single species of gp70 on each cell. Both gp70s were of the RM-MuLV class, which was compatible with the supposition that specific interference was responsible for the RM-MuLV resistance of these two cell lines. The specific molecular origins of the RM-MuLV like gp70s are as yet unknown. However, in at least one mouse strain, a preexisting endogenous RM-MuLV class gp70 gene was described [1].

H. Signal Hypothesis Models for Viral and Virus-Free Mouse Lymphomas

The known factors required for viral leukemogenesis in the mouse are the presence of an E-MuLV, the generation of an RM-MuLV, and an expanded population of cells of the T-cell lineage. Viral gp70 is known to stimulate blastogenesis, and this response is known to be critical for and predictive of development of leukemia [18]. The central element is that within the reactive nonspecifically expanded population, a cell occurs with a specific receptor for a single type of RM-MuLV gp70. Such cells have been described. When this cell becomes infected with the matching RM-MuLV, it produces both the driving signal

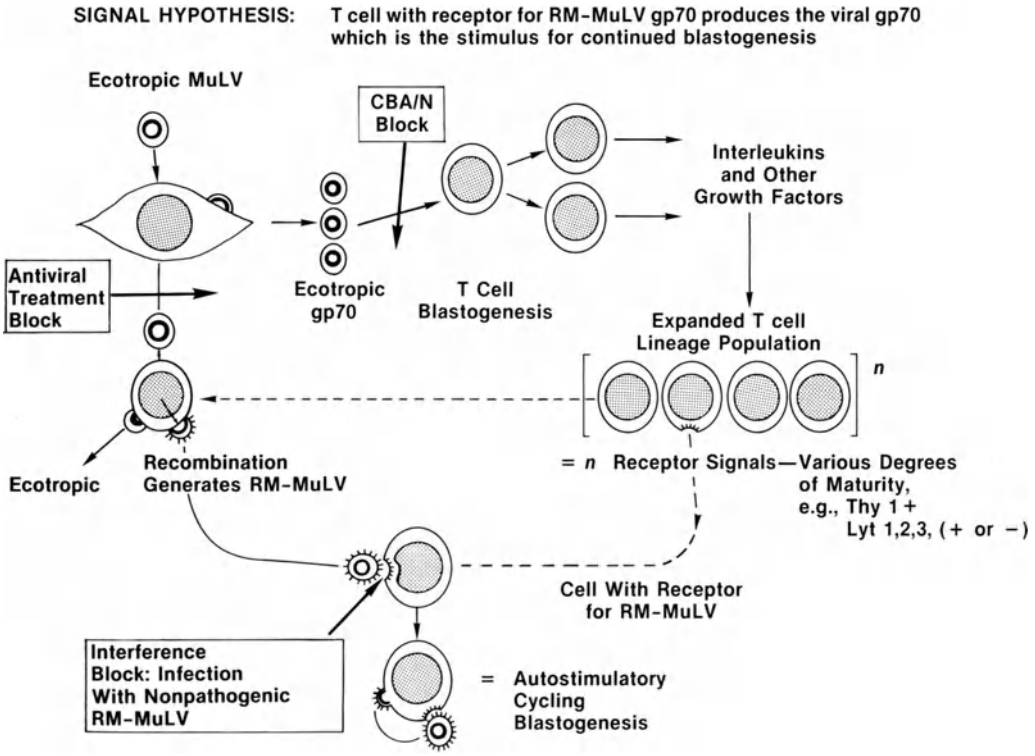

Fig. 2. A model describing multiple factors required for development of murine leukemia. Ecotropic MuLV is depicted with a smooth coat, RM-MuLV with projections. Ecotropic virus first replicates in various lymphoid organs in null cells and only later in T cells. CBA/N "block" describes the inability of lymphoid cells of that mouse strain to react in blastogenesis after exposure to ecotropic gp70, although the ability to generate new E and RM-MuLV appears not to be impaired. Interference "block" refers to the possibility that less pathogenic strains of RM-MuLV could preinfect the target cell which is then refractory to infection with the more pathogenic variant

and the receptor for it, resulting in a clonal population of autostimulated cells. This view, presented in Fig. 2, is different in principle from the Weissman model, which presupposes anergy to endogenous ecotropic MuLV gp70 [20].

The etiology of virus-free mouse lymphoma may be the more important example of the hypothesis. The ultimate requisite signal would be only an RM-MuLV gp70. Recombinational events could occur within the genome, involving whole or partial retrovirus-like gp70 sequences after chemical or physical perturbation of DNA. Whole virus need not be produced. However, if such recombination generates a surface gp70 in a cell with a receptor for it, such a cell could be capable of cycling blastogenesis resulting in an unlimited clonal expansion. The presence of loosely attached, unique, single species of an RM-MuLV like gp70 in virus-free lymphomas of several mouse strains would tend to support such a view [11].

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Expression of Retroviruses During Early Mouse Embryogenesis *

R. Jaenisch, K. Harbers, D. Jähner, C. Stewart, and H. Stuhlmann

A. Introduction

To study mechanisms of gene regulation involved in embryonal development, we inserted the Moloney leukemia virus (M-MuLV) genome into the germ line of mice. Thirteen different substrains of mice were obtained, each carrying one single copy of the Moloney leukemia virus as a Mendelian gene [1–3]. These substrains differ in their genotype (different chromosomal integration sites; *Mov* loci) as well as in their phenotype of virus expression: the majority of substrains exhibit no virus expression at all, and four substrains express virus at different stages of development. In Table 1 the characteristics and the time of virus activation during development in the different *Mov* substrains are summarized. Recent evidence obtained in our laboratory [4] indeed suggested that tissue-specific activation of viral genomes carried in the germ line of mice may be regulated by similar mechanisms, as has been proposed for the tissue-specific activation of developmentally regulated genes [5]. Our results furthermore suggested that the chromosomal position at which virus integration occurred influenced the timing in development and the cell type where the proviral genome became activated [2, 3].

As a means of studying the underlying regulatory mechanisms we have analyzed the extent of DNA methylation [6–8] of the viral genomes. All proviral genomes carried in the *Mov* substrains were highly methylated, were not expressed in the tissues tested, and were not infectious in a transfection assay [9]. However, when the methyl groups were removed by molecular cloning of the proviral copies, they were rendered highly infectious [10]. These results strongly suggested that DNA methylation plays a causative role in gene regulation during development and differentiation.

The *Mov* substrains were derived by exposing preimplantation mouse embryos to M-MuLV. Since the infecting retroviral DNA was not methylated, de novo methylation of the proviral genomes must have occurred at some point either during development of the infected embryo and/or as a consequence of their transmission through the germ line. Furthermore, it has been shown previously that early mouse embryos as well as embryonal carcinoma (EC) cells [11–14], which have many features in common with embryonic ectoderm cells of early mouse embryos, are nonpermissive for replication of M-MuLV. The experiments summarized in this review article were performed to understand the parameters that prevent expression of viral genomes introduced into early embryos and to correlate this with DNA methylation.

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Strain	M-MuLV sequences Genetic locus	Expression of virus		Other characteristics
		Viremia	Time of activation	
BALB/c	*Mov*-1	+	1 week after birth	Virus on chromosome 6
ICR	*Mov*-2	+	In 20% as adults	
	Mov-3	+		
	Mov-4	–		del. env.
129	*Mov*-5	–		
	Mov-6	–		del. env.
	Mov-7	–		
	Mov-8	–		
	Mov-9	+		
	Mov-10	–		
	Mov-11	–		
	Mov-12	–		
C57BL	*Mov*-13	+	During embryogenesis	"Gray hair"

Table 1. Mouse strains with germ line integrated Moloney leukemia virus

The table summarizes the characteristics of mouse substrains carrying M-MuLV in their germ line. For details, see [3]

B. Results

Two experimental approaches were used to investigate the molecular parameters that prevent expression of RNA tumor viruses in embryonal cells. The fate of the infecting viral DNA was directly followed and compared in tissue culture by infecting pluripotent EC cells or differentiated cells. In a second approach the preimplantation or postimplantation mouse embryos were exposed to M-MuLV, and viral genomes carried in the adult animals derived from the respective infected embryos were characterized. In both experimental approaches the expression of viral genomes was studied by the XC plaque assay, quantitative RNA hybridization, and/or in situ hybridization, and modifications of the viral genomes were characterized by restriction enzyme analysis and by transfection assay of the high molecular weight DNA. The results of these experiments have been published [15, 16] and will be briefly summarized in Tables 2 and 3.

Cells	Time after infection	Expression	Presence of unintegrated DNA	Methylation	Infectivity
EC	24 h		+	–	+
cells (F-9)	4 weeks	–	–	+	–
3T3	24 h	+	+	–	+
EB22/20	4 weeks	+	–	–	+

Table 2. De novo methylation of M-MuLV genomes after infection

Pluripotent F-9 cells or differentiated derivatives were exposed to virus and analyzed for virus expression, DNA methylation, and infectivity as described in [15]

Time of exposure	Virus expression after infection	M-MuLV in adult	
		Methylated	Infectious
Preimplantation stage (1 – 16 cells)	–	+	–
Postimplantation stage 10^4 – 10^6 cells	+	–	+

Table 3. De novo methylation of M-MuLV after infection of mouse embryos

Embryos were exposed to virus at different stages of embryogenesis. DNA methylation and infectivity of the M-MuLV genomes carried in the adult animals were analyzed as described in [16]

I. Infection of Embryonal Carcinoma Cells with M-MuLV

Pluripotent EC cells (F-9 cells) and differentiated cells (EB22/20, a differentiated derivative of EC cells or NIH 3T3 cells) were exposed to M-MuLV [15]. Whereas virus replicated efficiently in the latter cells, as revealed by infectious center assay or RNA hybridization experiments, no virus expression was found in F-9 cells (Table 2). The following experiments were performed to study the block in virus expression in F-9 cells.

The kinetics of virus integration were established and indicated that all viral genomes integrated stably into the cellular chromosomal DNA during the first 2 or 3 days after exposure of cells to M-MuLV. Analyses using methylation-sensitive restriction enzymes revealed that viral DNA in F-9 cells remained unmethylated as long as it was in the episomal state but became de novo methylated soon after chromosomal integration. This correlated well with the transfection assay: DNA isolated early after infection was biologically active, whereas DNA isolated late when free viral DNA was no longer present failed to induce XC plaques upon transfection (Table 2). The methylated proviral copies, however, were potentially infectious because they induced XC plaques when the recipient cells for transfection were treated with azacytidine. This drug is believed to interfere with maintenance methylation. In contrast, viral genomes introduced into EB22/20 or NIH 3T3 cells remained unmethylated as well as infectious after chromosomal integration (Table 2).

Our results strongly suggest that expression of proviral genomes introduced into pluripotent EC cells is suppressed upon chromosomal integration and that this inactivation can be correlated with de novo methylation of the viral DNA.

II. Infection of Mouse Embryos with M-MuLV

Due to technical problems in obtaining sufficient material, the fate of viral DNA introduced into early mouse embryos could not be analyzed directly in a similar way to that described above for the tissue culture systems. Therefore viral genomes were studied in adult animals derived from the infected embryos. Two stages of embryogenesis, which differ fundamentally in their response to virus infection, were compared: (a) Embryos were infected with virus at the preimplantation stage, a stage at which no viral expression takes place [11, 14]; (b) embryos were microinjected with virus at day 8 of gestation. At this stage efficient virus replication occurs in cells of all tissues as revealed by in situ hybridization [16] or by analyzing the tissue distribution of viral DNA and RNA in the adult [17]. The results of analyzing the modification and infectivity of the viral genomes carried in the adults are summarized in Table 3 [16]. Restriction enzyme analysis revealed that copies introduced into preimplantation embryos became de novo methylated and remained highly methylated throughout the life of the animal, whereas viral genomes introduced 5 days later into the postimplantation embryo remained unmethylated. The results of transfection assays con-

firmed these results. DNA derived from animals exposed to virus at the postimplantation stage was highly infectious, in contrast to DNA from animals exposed to virus at the preimplantation stage.

These observations extend the results obtained in vitro with EC cells to the in vivo situation. They suggest that an efficient de novo methylation activity is a characteristic of totipotent early embryos and may be involved in the inhibition of viral gene expression. Neither de novo methylation activity nor inhibition of virus replication, however, is observed at day 8 of development.

C. Conclusions

The introduction of foreign cellular and retroviral genomes into early mouse embryos has been used as a means of investigating the regulation of gene expression in mammalian development [18–23]. The results obtained in our system established that both embryonal carcinoma cells and preimplantation mouse embryos are nonpermissive for expression of retroviral genomes. Retroviruses introduced into differentiated derivatives of EC cells or into postimplantation mouse embryos at day 8 of gestation, however, were able to replicate efficiently. This defines a switch of early differentiating cells in their ability to support retroviral expression which is developmentally regulated.

The switch in gene expression was correlated with efficient de novo methylase activity in pluripotent cells. Retroviral genomes introduced into EC cells or into preimplantation mouse embryos became efficiently de novo methylated, in contrast to viral genomes introduced into differentiated cells or into postimplantation embryos. The results with EC cells indicated that this enzyme activity de novo methylates viral genomes only after chromosomal integration and does not act on DNA molecules which are in the episomal state. This is relevant to the observation that DNA microinjected into mouse zygotes [24] or into *Xenopus* eggs [25] is expressed as long as it remains in an episomal state. In addition, unintegrated DNA injected into *Xenopus* eggs was shown to remain unmethylated [26]. Our results furthermore show that the maintenance methylation activity is faithful in preserving the respective methylation pattern of the proviral genomes throughout the life of the animal. The de novo methylation activity in embryonal cells may be of general significance as not only viral but also cloned globin DNA, which was microinjected into mouse zygotes, became de novo methylated (F. Costantini and E. Lacy, personal comminication).

If the de novo methylation activity in embryonal and efficient maintenance methylation in later cells are involved in repression of proviral genomes, what is the origin of infectious virus in mice derived from preimplantation embryos exposed to virus? Because virus, once activated, will infect all susceptible cells and spread throughout the animal, demethylation and activation of virus at a given stage of development and in a specific, as yet unidentified, population of cells would be sufficient to lead to viremia. Demethylation of a given provirus in specific cells may depend on the chromosomal position where integration took place, and proviral genome activation may thus be regulated by similar mechanisms as has been proposed for the tissue-specific activation of developmentally regulated genes [5, 27]. Gene activation of the proviral genome in *Mov-1* mice appears to be compatible with such a hypothesis [4].

Our results suggest that embryonal cells may possess an efficient de novo methylation activity that inactivates any DNA which is introduced into the early embryo. This may have evolved as a mechanism to protect the developing embryo against deleterious consequences of virus infections. Finally, our results pose intriguing questions concerning the control of gene expression during early development, and it will be of great interest to study the methylation of genes that are active in preimplantation embryos and in embryonal carcinoma cells.

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Natural History of M-MSV Tumors in Mice Carrying Endogenized Moloney Leukemia Virus *

L. Chieco-Bianchi, E. D'Andrea, A. De Rossi, P. Zanovello, F. Ronchese, and D. Collavo

A. Introduction

The conservation of endogenous type C DNA provirus sequences throughout evolution has raised the question of whether or not they exert normal physiological functions. Among the different hypotheses advanced has been suggested that a genetically transmitted virus could serve to protect the host, possibly via immune reactions, against related, more virulent viruses that may be acquired from the outside [14]. No substantial evidence for this hypothesis has been provided, however. Years ago during studies aimed at investigating in vivo interaction between endogenous and exogenous type C murine retroviruses, we noticed that AKR mice which had been injected with Moloney murine sarcoma virus (M-MSV) developed tumors with a longer latent period than that observed in mice of conventional strains. Furthermore, these late appearing tumors showed an unusual growth pattern, which was characterized by a slow but continuous progression until the host's death [2]. Subsequently, these findings were confirmed in larger studies using different mouse strains [4, 7, 8]. Figure 1 depicts the general pattern of tumor behavior that has emerged; mice characterized by early endogenous ecotropic virus activation are resistant to early M-MSV oncogenesis, but late appearing progressive tumors are observed in the majority of strains. While late tumor progression is probably due to immunological tolerance of cytotoxic T-lymphocytes (CTL) toward virus-coded antigens [6], the mechanism underlying resistance to early tumor induction is still poorly understood. BALB/Mo mice, which carry the exogenous Moloney leukemia virus (M-MuLV) as an endogenous virus integrated at a single locus (*Mov-1*) on chromosome 6 [1, 11], offer the unique opportunity of studying whether the full expression of genetically transmitted M-MuLV confers resistance against the antigenically related (M-MuLV) M-MSV complex. Indeed, in a first series of experiments [9], it was found that the natural history of induced tumors in these mice is quite similar to that observed in AKR type mice. In addition, resistance to early tumors appears related to the time course of M-MuLV activation as well as to the presence in the serum of normal mice of antibodies possessing specific binding capacity to M-MuLV surface determinants. The possibility that this particular tumor pattern might be influenced by these antibodies, or by virus-specific CTL activity, is discussed in this study.

B. Results and Discussion

I. Biological Activity of Natural Antibodies

The detection of natural antibodies specific for M-MuLV in the serum of adult BALB/Mo mice by means of a ^{125}I-labeled protein A binding assay prompted us to investigate its possible role in M-MSV oncogenesis.

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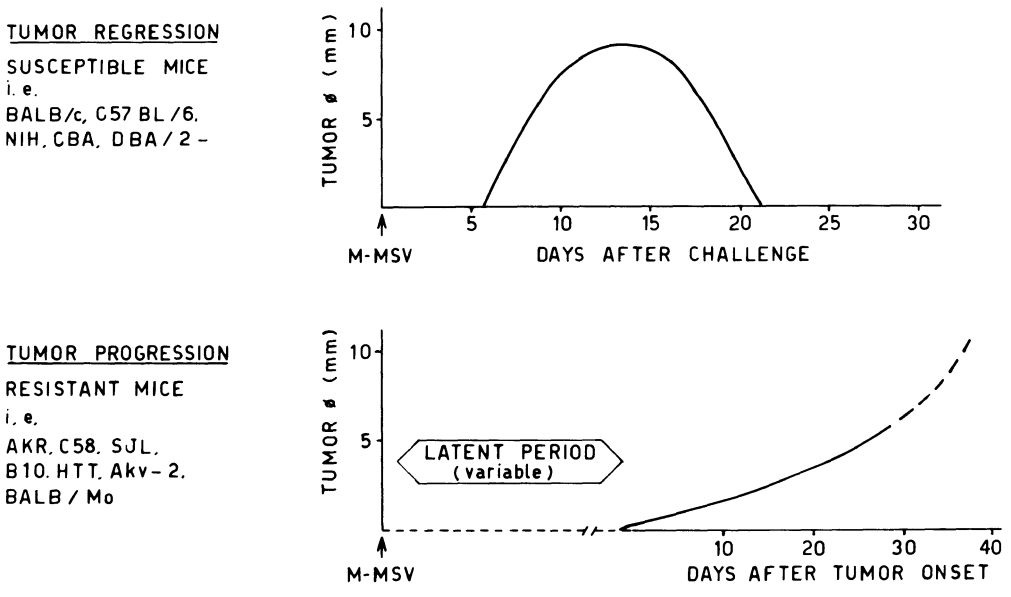

Fig. 1. Patterns of tumor behavior following M-MSV inoculation in adult mice of different strains

We first assayed the binding capacity of serum pooled from 3-month-old BALB/Mo mice on SC-1 mouse cells chronically infected with M-MuLV by immunofluorescence, using sera from normal or M-MSV injected adult BALB/c mice as controls. As shown in Table 1, immunofluorescence positive (IF+) cells were detected with BALB/Mo serum and with M-MSV immune BALB/c serum, while cells incubated with normal BALB/c serum were negative. Thus, BALB/Mo serum apparently pos-

Table 1. Reactivity of BALB/c and BALB/Mo sera on M-MuLV chronically infected SC-1 cells as evaluated by immunofluorescence assay[a, b]

Serum sample tested	Serum dilution		
	1:10	1:20	1:40
BALB/c	0.1	0.1	0.1
BALB/Mo	50 – 60	30 – 50	10 – 30
BALB/c-M-MSV[c]	80 – 100	60 – 80	50 – 60

[a] The immunofluorescence assay was performed by incubation of acetone-fixed cells with serum from tested mice followed by incubation with goat anti-mouse Ig fluorescinated serum (National Cancer Institute, Bethesda, USA) at 1:40 dilution

[b] Values refer to the percentage of immunofluorescence-positive cells

[c] Donor mice were immunized by i.m. injection of M-MSV (2×10^6 FFU/0.10 ml), and subsequently i.p. boosted twice at 2 – 3 week intervals with the same virus dose

Table 2. Virus neutralization by sera of normal and M-MSV injected BALB/c and BALB/Mo mice[a]

Serum sample tested	Serum dilution		
	1:20	1:40	1:80
BALB/c	0.01	0.01	0.01
BALB/Mo	20.60	7.60	0.01
BALB/c-M-MSV[b]	98.25	84.30	52.70
BALB/Mo-M-MSV[b]	26.80	9.80	0.01

[a] The assay was performed by M-MSV focus reduction on SC-1 cells as reported [10]; 200 FFU/0.20 ml were incubated with serial dilutions of sera at room temperature for 1 h before culture infection. Values refer to the percentage of neutralization calculated as $=[1-(Vn/Vo)] \times 100$

[b] See Table 1 footnote c

Table 3. Blocking of virus neutralization by normal BALB/Mo serum[a]

Serum sample tested	Serum dilution		
	1:30	1:60	1:120
BALB/c-M-MSV	97.80	74.80	44.50
BALB/c-M-MSV + BALB/c	98.00	73.40	44.00
BALB/c-M-MSV + BALB/Mo	91.00	49.70	11.20

[a] Blocking of neutralization was carried out by incubating for 1 h at room temperature M-MSV with pooled sera, at 1:20 dilution, from normal, 2-month-old, BALB/c or BALB/Mo donors, followed by an additional 1 h incubation with two-fold serial dilutions of BALB/c-M-MSV immune serum (see Table 1, footnote c). Values refer to the percentage of neutralization calculated as $=[1-(Vn/Vo)] \times 100$

sesses binding capacity for M-MuLV induced cellular antigens as well.

We then performed virus neutralization assay by evaluating M-MSV focus reduction on SC-1 cells. Table 2 shows that, compared to M-MSV immune BALB/c serum, very little if any neutralizing activity was exerted by normal BALB/Mo serum and, more interestingly, by the putative M-MSV immune BALB/Mo serum. However, preincubation of M-MSV with normal BALB/Mo serum, followed by incubation with M-MSV immune BALB/c serum, remarkably reduced the neutralizing activity of the latter (Table 3). This finding is similar to that observed with monoclonal antibodies specific for gp52 of mouse mammary tumor virus [13], and suggests that natural antibodies found in BALB/Mo mice may recognize viral antigenic determinants distinct from, but adjacent to, the target site for neutralizing antibody. Thus, the observation that BALB/Mo serum binds to M-MuLV and yet competes with neutralizing activity of M-MSV immune serum would indicate its potential blocking capability. Indeed, in preliminary experiments, facilitation in M-MSV tumor growth was observed in BALB/c mice treated with repeated injections of normal BALB/Mo serum.

II. Generation of Virus-Specific CTL

We have repeatedly observed that M-MuLV neonatally injected mice, challenged as young adults with M-MSV, do not regress their sarcoma and are unable to generate virus-specific CTL [3, 5, 6]. Since activation of endogenous M-MuLV in BALB/Mo mice takes place only after 1–2 weeks of postnatal life [9, 12], we considered it of interest to ascertain whether virus-specific CTL could be generated in adult BALB/Mo mice challenged with M-MSV.

However, since the BALB/Mo mouse line was originally derived from an M-MuLV infected (BALB/c × 129) blastocyst, the possibility that residual heterozygosity deriving from the 129 (H-2^b) strain might interfere with CTL activity due to a lack of H-2 restriction had to be considered. Accordingly, F1 mice were produced by mating BALB/c (H-2^d) females with BALB/Mo males; these F1 hybrids, albeit heterozygous for the *Mov-1* locus, are quite similar to the parental BALB/Mo mice as far as time course and levels of M-MuLV expression, and M-MSV tumor response are concerned (unpublished data). Therefore, spleen cells from BALB/c and (BALB/c × BALB/Mo)F1 donors previously injected with M-MSV were restimulated in vitro with LSTRA (H-2^d) Moloney leukemia or normal (BALB/c × BALB/Mo)F1 spleen cells, and then assayed on ^{51}Cr-labeled LSTRA targets. Table 4 shows the results of a typical experiment. Spleen cells from the M-MSV injected F1 mice did not lyse LSTRA leukemic targets, while those from BALB/c mice gave high cytotoxicity. Furthermore, restimulation of BALB/c effector cells with spleen cells obtained from 8-week-old normal F1 donors was highly effective in producing CTL activity, and demonstrated that F1 spleen cells express antigenic determinants relevant for CTL generation. Thus, in agreement with results obtained by infecting newborn mice with exogenous MuLVs [3, 5, 6], it appears that a state of immunological tolerance involving a CTL subpopulation is also present in F1 mice (and by inference in their BALB/Mo parent).

Table 4. CTL generation from spleen cells of BALB/c and (BALB/c×BALB/Mo)Fl mice injected with M-MSV[a]

Effector cells	Stimulator cells	Target cells	% specific ^{51}Cr release at effector/target cell ratio of		
			30:1	10:1	3:1
BALB/c	LSTRA	LSTRA	54	40	21
BALB/c	(BALB/c×BALB/Mo)Fl	LSTRA	68	35	25
(BALB/c×BALB/Mo)Fl	LSTRA	LSTRA	1	1	1

[a] Eight-week-old mice were splenectomized 14 days after M-MSV inoculation. Cytotoxic T-lymphocyte activity was evaluated after restimulation in vitro with LSTRA cells (a BALB/c transplantable leukemia originally induced by M-MuLV) or (BALB/c×BALB/Mo)Fl spleen cells pretreated with mitomycin C [5]

In conclusion, these data indicate that natural anti-M-MuLV antibodies found in BALB/Mo mice are not involved in resistance to early tumor induction by M-MSV, but instead, together with virus-specific CTL unresponsiveness, might be responsible for lethal evolution of the induced tumors.

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Induction of Histiocytomas by Pristane Treatment of Mice Chronically Infected with Moloney Murine Leukemia Virus *

P. Nobis and J. Löhler

Normally Moloney murine leukemia virus (M-MuLV) induces T-cell derived leukemia in mice after a latency period of several months. Recently this virus was shown to replicate in infected mice in a wide variety of cells of hemopoietic and non-hemopoietic origin without transforming these cells [1].

Here we report that histiocytic tumors were induced after pristane injection into the peritoneal cavity of mice chronically infected with M-MuLV. Two sets of viremic mice were used: At 8 weeks of age BALB/c mice were infected with M-MuLV and 2 and 4 weeks later they received 0.5 ml pristane intraperitoneally. In the second experiment BALB/Mo mice [2] were injected with pristane at 6 and 10 weeks of age. BALB/Mo mice carry the M-MuLV as an endogenous virus and are viremic from about 1 week of age on [3]. After a latency time of at least 2 months following the second pristane application, 6 of 14 M-MuLV infected BALB/c and 19 of 24 BALB/Mo mice developed abdominal tumors with massive hemorrhagic ascites. The surface of the peritoneum was covered with whitish solid layers of tumor tissue. The ascites fluid contained up to 10^8 cells/ml. In the thymus, spleen, and lymph nodes, growth of histiocytic tumor cells was not observed.

The tumors and the ascites fluid were characterized histologically and cytologically. The results are summarized in Table 1. The markers shown are characteristic for the monocyte/macrophage lineage; hence the tumors could be classified as histiocytomas.

Table 1. Histological and cytological characterization of tumors induced by pristane treatment of mice chronically infected with Moloney murine leukemia virus

Feature:	Intraperitoneal tumor	Ascites fluid/ tissue culture
Morphology:	Histiocytoma	Promonocytes/ monocytes/ Macrophage-Like cells
Reticuline	+	–[a]
α-*N*-esterase	+	+
Acid phosphatase	+	+
Lysozyme	+	+
α_1-antichymotrypsin	–	+
M-MuLV specific proteins	+	+

[a] Reticuline is only a consistuent of solid tissue

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When transplanted into syngeneic hosts most of the histiocytomas could be serially passaged. From nine tumors permanent tissue culture lines were established. With one exception all tissue culture lines release high titers of ecotropic M-MuLV.

The infection of mice with M-MuLV usually results in the induction of T-cell leukemia several months after the infection. M-MuLV lacks a transforming gene and the leukemia induction by this virus is still poorly understood. An abnormal proliferation of lymphocytes caused by the chronical virus infection is regarded as one of the possible mechanisms leading to leukemia [4]. In our case a similar chain of events may be responsible for the formation of histiocytic tumors. The application of pristane into the peritoneal cavity induces a constant proliferation of normal phagocytes. But virus infection is needed to set off the development of this specific tumor.

Acknowledgments

BALB/Mo mice were a gift of R. Jaenisch.

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Purification of a Reverse Transcriptase-like Protein from the Plasma of a Patient with Chronic Myelogenous Leukemia and Production of Monoclonal Antibodies

P. C. Jacquemin

A. Introduction

Some human leukemic cells have been shown to contain a cytoplasmic reverse transcriptase (RT) [1]. The RT from cells of acute myelogenous leukemia (AML) patients has been shown to be specifically neutralized by IgG from hyperimmune sera prepared against RT of simian sarcoma virus (SiSV) and of gibbon ape leukemia virus (GaLV) [2]. An RT-like polymerase was also isolated from other neoplastic tissues, including the spleen of a child with myelofibrosis [3] and some melanoma tissue [4]. We have previously reported the presence of surface immunoglobulin on cells of patients with chronic myelogenous leukemia (CML) in blast crisis (BC) that specifically neutralized RT from feline leukemia virus (FeLV), and of surface IgG on AML and on some normal blood cells that preferentially reacted with RT from SiSV and GaLV [5, 6]. These membrane-bound IgGs were recovered by spontaneous release in the medium after overnight incubation. Since we found it difficult to identify a RT-like antigen on the surface of fresh leukemic cells, we looked at the plasma of a CML-BC patient for the presence of a RT-like antigen.

B. Results

I. Purification of a Protein with Chromatographic Properties of RT

A large quantity of plasma was obtained by repeated leukophoresis from a patient in CML-BC. Forty milliliters of it was supplemented with Triton X100 (0.5%), DTT (0.1 m*M*), NaCl (0.3 *M*), Tris HCl (0.05 *M*) pH 7.9, and PMSF (0.2 m*M*) and chromatographed on a DEAE-Agarose column to remove the nucleic acids. After a threefold dilution, the flow through was incubated for 10 min with 50 μ*M* polyguanilic acid [poly (G)] and 0.5 m*M* $MnCl_2$ and applied again to DEAE-Agarose following a procedure described by Sarangadharan et al. [7]. The 0.3-*M* eluate which did not possess enzymatic activity was iodinated and precipitated with different hyperimmune sera raised against type C virus proteins. A rabbit anti-Rauscher murine leukemia virus (R-MuLV) RT antiserum and an anti-FeLV p15 antiserum precipitated preferentially a protein of 74 kilodaltons. This protein was precipitated neither by a normal rabbit serum, nor by rabbit sera raised against R-MuLV gp70, R-MuLV p30, Baboon endogenous virus (BaEV) RT, and SiSV RT. In addition, goat antisera raised against SiSV gp70 or against human albumin did not precipitate the protein. The antiserum raised against R-MuLV RT appears very cross-reactive in enzyme neutralization assays; it neutralizes both R-MuLV RT and BaEV RT almost equally well. The antiserum against FeLV p15 appears to recognize p15E.

II. Production of Monoclonal Antibodies Against the 74K Protein

Balb/c mice were immunized subcutaneously with the crude preparation of RT-like antigen. Four weeks later, the spleen cells of the immunized mice were fused with SP/2 drug marked, non-immunoglobulin secreting myeloma cells [8].

Hybrid clones obtained were tested in a solid-phase radioimmunoassay for production of antibody recognizing the immunizing preparation. The iodine-labeled preparation of immunizing antigen was precipitated with the positive monoclonal antibodies. Two-dimensional electrophoresis of the immune precipitates showed that they precipitated the same 74K protein as the anti-R-MuLV RT and the anti-FeLV p15 antisera.

III. Further Purification of the RT-Like Protein

Taking advantage of the specificity of the monoclonal antibodies, an affinity column was prepared to further purify the antigen. From 30 mg crude RT-like antigen described above, 1.4 mg purified protein was eluted from the affinity column. The purified protein was labeled with ^{125}iodine and shown to be a clean preparation of a 74K protein by SDS polyacrylamide gel electrophoresis. It was also precipitable up to 100% by the rabbit anti-FeLV p15 antiserum and up to 80% by the anti-R-MuLV RT antiserum. The monoclonal antibody used to prepare the column also precipitated 100% of the labeled antigen. A monoclonal antibody against P15E (9) precipitated the protein up to 60% but with a low titer. The precipitation of the antigen could be competed by virus cores (R-MuLV, GaLV) and by purified FeLV RT (the only one tested in competition) but not by pure R-MuLV p30. The purified antigen still bound to poly (G) in the presence of $MnCl_2$ and when introduced in a RT assay competed with the active enzyme for the template primer dT_{12-18}-poly rA.

C. Conclusion

We purified a 74K protein with chromatographic properties like RT but no enzymatic activity from the plasma of a patient with CML-BC. After labeling with ^{125}iodine the protein was precipitable by a broadly cross-reactive antiserum raised against R-MuLV RT and by a rabbit antiserum raised against FeLV p15. Monoclonal antibodies were developed that recognized specifically the 74K protein. They allowed us to purify the protein further. We found that the purified protein still had affinity for poly (G) and could compete for the template primer with active enzymes in an RT assay. When labeled the pure protein could also be precipitated by monoclonal antibodies. We are currently investigating whether this protein is expressed on the surface of cells from CML patients in BC, for which we have previously demonstrated the presence of immunoglobulin with anti-RT activity.

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Detection of Group and Interspecies Reactivities in Mammalian C-Type Virus p30 Proteins and Corresponding Human Antigens *

H. Schetters, V. Erfle, and R. Hehlmann

A. Introduction

We previously reported the presence of RNA tumor virus related antigens in human leukemic sera. The antigens were detected by the ELISA method in 30%–40% of patients with acute leukemia [1, 2]. Since these antigens from human sera that cross-react with primate C-type viral p30 protein had been observed with the ELISA method and since retroviral group and interspecies reactivities had been previously characterized mostly by competition radioimmunoassay, we examined the applicability and reliability of the ELISA method for the detection of group and interspecific determinants in p30 proteins of mammalian C-type retroviruses [4].

The specificity of the observed reaction was further demonstrated by competition ELISA, by preabsorption of the coating antibody with homologous and heterologous antigen, and by ELISA with $F(ab')_2$ fragments of the IgG molecules [5].

After exclusion of rheumatoid factor (RF) interference, the immunoaffinity purified human antigen was characterized by SDS polyacrylamide gel electrophoretic analysis, enzymatic digest, and peptide maps. The antigen found in human leukemic sera is a protein distinct from common serum proteins.

B. Results

I. Specificity of the Test System

To determine the specificity of the ELISA technique five different approaches were chosen: specificity of the antisera used, competition ELISA, preabsorption test, ELISA with $F(ab')_2$ fragments of IgG, and interspecies reactivities detected by the ELISA technique.

1. Specificity of the Antisera

For the search for human antigens, antisera against three different RNA tumor viral p30s (MuLV p30, SiSV p30, and BaEV p30) were used. To establish their specificity for the homologous p30, they were tested with purified p30 proteins, disrupted whole viruses, and virus-producing cell lysates. It was shown that the antisera recognized the homologous antigen very well, while the heterologous antigens bound only to a very limited extent [3].

2. Competition ELISA

In this assay the antiserum was incubated with increasing amounts of homologous or heterologous viral p30, virus, extracts of virus-producing cells, or human sera. The remaining binding activity to the homologous p30 protein was measured. The human sera competed the binding partially in the anti SiSV p30 system while homologous viral protein blocked the antibody activity completely (Fig. 1).

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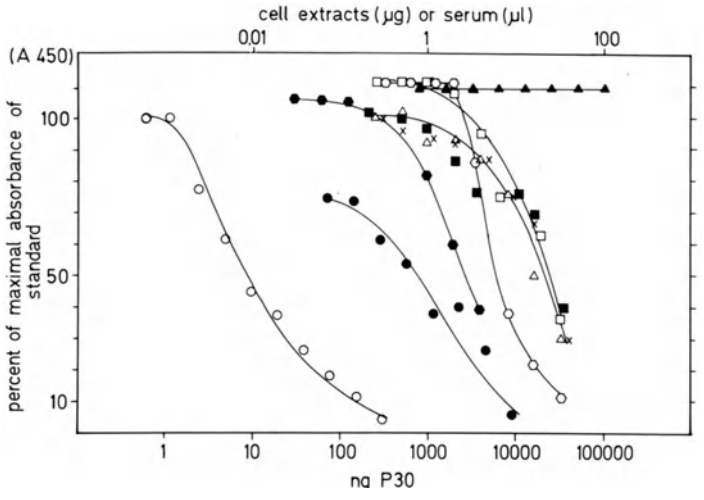

Fig. 1. Competition of human sera for SiSV p30 measured by the competition ELISA. The plate was coated with SiSV p30 (30 ng/ml) and the antiserum was preabsorbed with increasing amounts of antigen or human serum. The competing antigens were: ○ SiSV p30; ● SiSV-71AP1 cell extract; ■ L175, ALL; □ L153, CML-BC; × L148, CML; ⬡ L183, ALL; ⬢ L40, AProl; △ Mult.Myel.; ▲ FCS, HSA, MuLV inf. C3H cell extract, BaEV inf. A204 cell extract

3. ELISA After Preabsorption of Coating Antibody with Homologous p30 Protein

Identical results to the C-ELISA assay were achieved, when the coating antibody for an antigen test was preabsorbed with the homologous p30 protein. Corresponding to the increasing amount of absorbent the binding of the homologous p30 and of the human sera tested decreased, thus confirming the specificity of the test system.

4. Preabsorption Test

The specificity of the test was further analyzed by using $F(ab')_2$ fragments of the anti SiSV p30 and anti BaEV p30 IgG molecules. The binding activity of the fragments was decreased to some extent for the homologous antigens and for the human antigens compared to undigested IgG molecules. But a specific binding of homologous antigen and human antigen remained while sera of healthy donors and animal sera showed no binding activity (Fig. 2).

5. Interspecies Reactivities Detected by the ELISA Technique

For the determination of the recognition of interspecific reactivities by the ELISA method, four groups of C-type retroviruses (MuLV, FeLV, SiSV/GaLV, and BaEV/RD114) were assayed for group-specific and for interspecific reactivities of their p30 proteins. We found that the ELISA can detect group-specific as well as interspecies

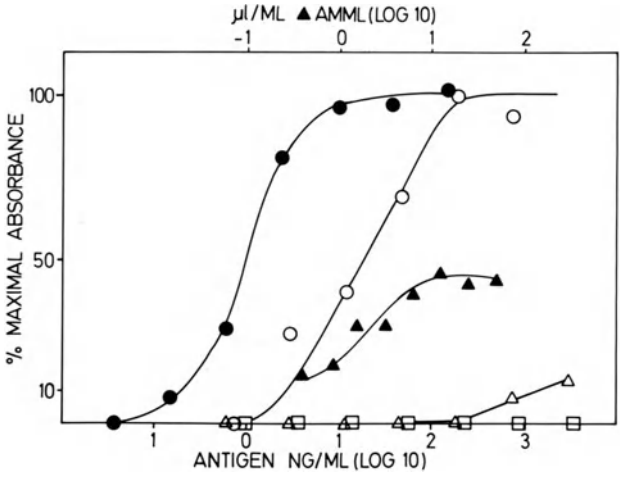

Fig. 2. Binding of homologous and heterologous antigen and one human leukemic serum to $F(ab')_2$ fragments of anti-SiSV p30 IgG detected by the ELISA technique. Antigens used were: ● SiSV p30; ○ SiSV virus; □ BaEV virus; △ MuLV virus; ▲ L117, AMML serum

determinants sensitively and reproducibly in purified p30 proteins, disrupted viruses, and cell extracts if an anti p30 multispecies antiserum is used [4]. When monospecific antisera against MuLV p30, SiSV p30, or BaEV p30 were used only specific reactivities were detected reproducibly, whereas the detectability of interspecies determinants depended on the antisera used. Comparing native and SDS denatured p30 proteins the detectability of the denatured MuLV p30 was better than that of the native MuLV p30, suggesting that some of the reactive determinants are localized inside the protein molecule and are freed by the denaturation process (Table 1).

II. Characterization of the Purified Human Antigen

For the characterization of the human antigens (a) the nature of the antigen (b) the molecular weight, and (c) peptide maps were determined.

1. The Nature of the Human Antigen

To determine the nature of the human antigen from a selection of human sera (Table 2) that were isolated by immunoaffinity chromatography, they were subjected to digests with five different enzymes (RNAase A, DNAase I, lipases, mixed

Table 1. Recognition of group and interspecies determinants in purified p30 proteins, disrupted viruses, and cell extracts with antisera against native p30 proteins

Antisera	Anti-MuLV p30	Anti-SiSV p30	Anti-BaEV p30	Anti-p30 interspecies
Antigens				
EV-purified p30				
MuLV	1.2[a]	> 2,500	> 2,500	1.1
SiSV	> 214	0.55	214	1.7
BaEV	> 3,400	> 3,400	4.4	3.6
FeLV	2,400	> 2,500	> 2,500	NT
SDS gel purified p30				
MuLV	1.3	340	1,900	4.1
SiSV	> 2,100	< 0.38	> 2,100	44
BaEV	> 1,300	600	2.8	2.1
FeLV	> 170	> 170	> 170	0.8
GaLV	> 1,000	10.0	> 1,000	44
RD114	90	> 450	450	1.7
Viruses				
MuLV	3.2	2,300	> 22,000	< 0.8
SiSV	3,800	< 2.0	> 32,000	8.5
BaEV	> 23,000	> 6,000	2.7	< 0.85
FeLV	> 18,000	> 18,000	> 18,000	< 0.66
GaLV	> 32,000	3.2	> 32,000	10.0
RD114	> 48,000	> 48,000	6,700	< 3.5
Cells				
A7573	> 100,000	> 100,000	> 100,000	> 100,000
CCL88	> 96,000	> 96,000	> 96,000	> 96,000
MuLV-Balb/c spleen	115	1,600	> 108,000	1,800
SiSV-71AP1	6,000	43	> 96,000	1,800
M7BaEV-A204	> 100,000	40,000	900	950
FeLV-A7573	> 100,000	25,000	> 100,000	1,100
GaLV-CCL88	> 100,000	1,700	> 100,000	22,000

EF, electrofocusing; NT, not tested

[a] Numbers are nanograms of proteins to reach 20% of maximal binding

Table 2. Crossreacting antigens in human leukemic serum

Sera/follow-up sera of same patient	Diagnosis	Crossreaction with	
		SiSV p30	BaEV p30
		in ng equivalents/ml	
L22/L23/L107/L117	AMML	>600/>600/>600/>600	>1840/>1840
L99	AMML	30	16
L80/L96/L119	AMML	80/9.6/0	>1840/78/9.6
L88	AMML	14	170
L39	AML	>600	>1840
L29/L124	AML	72/>600	0
L130	AML	>600	>1840
L102	AML	8.5	1040
L150/L151	AML	12.4/13.2	NT
L101	AML	0	8
L40/L66/L69	AProL	6.4/11/0	NT
L73	ALL	14	0
L183	ALL	70	NT
L175	ALL	60	NT
L74/L75/L149/L158	CML	14/8.4/18/6.4/10	0
L159	CML-BC	8.8	NT
L163	CML-BC	8.8	NT
L83	CML-BC	15	>1840
L98	CML-BC	>600	>1840
L103	CML-BC	0	4.8
L82	CML-BC	6.8	18.4
L105	CML-BC	5.6	6.8
L153	CML-BC	19.4	NT

AMML, acute myelomonocytic leukemia; AML, acute myeloblastic leukemia; AProl, acute promyelocytic leukemia; ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia; BC, blast crisis; NT, not tested

glycosidases, or protease). From these five enzymes only the protease degraded the antigen as could be judged by SDS polyacrylamide gel electrophoretic analysis and by measuring remaining binding activity in the ELISA.

2. Determination of the Molecular Weight

On SDS polyacrylamide gel the antigen showed mainly one band. From its position, compared to BSA, it has an approximate molecular weight of 70,000 daltons.

3. Correlation of the Human Antigen to SiSV p30 and BaEV p30 by Peptide Mapping

The relatedness of the human antigen to the primate viral p30 proteins was determined by peptide maps. The peptide maps of the antigens isolated from two different patients were compared to each other, to SiSV p30 or BaEV p30, and to common human serum components like human serum albumin, transferrin, fibrinogen, plasmin, immunoglobulins G and M, and α-fetoprotein. The peptide patterns of the human antigens are identical and revealed homologies to SiSV p30 or BaEV p30 of 50%–60% of the number of p30 peptides. To the serum components the peptide homology reached up to 25% what is within the background range of specificity.

C. Discussion

It was possible to recognize specifically antigens in human sera with the ELISA

method that crossreact with primate C-type viral proteins. The ELISA method was previously shown to recognize specifically the homologous antigen and also to detect interspecies reactivity, which is crucial for the recognition of human crossreacting antigens. To show that these reactions, despite of broad reactivity, remain specific for the viral p30 proteins further supporting tests were undertaken. C-ELISA and ELISA with preabsorbed antibody or $F(ab')_2$ fragments of IgG all showed that the reaction was specific, excluding interfering reactions like rheumatoid factor or artifactual binding to other determinants of the IgG molecule rather than to the antigen binding site.

The antigen seen in human sera with antiserum against primate C-type viral p30 are proteins with a molecular weight of 70,000 daltons. From other publications, however, it is known that incomplete processing may lead to an accumulation of precursor proteins in the cell, virus-coded proteins like reverse transcriptase or *onc* proteins should be found to be present in this molecule. Another possibility would be that this protein is a fusion protein of virus-related p30 protein and cellular protein, as has been shown for some transformation proteins that were first detected in the avian leukemia virus system with anti p30 protein. In the first case it should be possible to detect other viral proteins in this molecule and in the second case transformation specific reaction like protein kinase activity must be looked for. The first possibility is favored by Jacquemin (this volume), who found in a leukemic patient a protein with a molecular weight of 74,000 daltons, reactive with anti MuLV reverse transcriptase that besides the reverse transcriptase determinants also contains feline leukemia virus p15 determinants. That we can find fusion proteins might be a sign for the presence of RNA tumor viruses, even when infectious viruses cannot be isolated.

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Papovaviruses and Human Tumors

H. zur Hausen

Papovaviruses contain the two subgroups "polyomaviruses" and "papillomaviruses" (reviewed in [20]). Members of both subgroups are clearly oncogenic, most notably the papillomaviruses, which induce papillomas within their natural hosts (see review [24]). Viruses of both subgroups can be distinguished morphologically, biochemically, and biologically. The polyomaviruses are nonenveloped, eicosaedral particles of 40 nm containing a circular double-stranded DNA molecule of about 3.3×10^6 daltons. The papillomaviruses show similar structural features. They are, however, larger in size (50–55 nm) and contain a DNA molecule of about 5.0×10^6 daltons. The structural organization of the genome is totally different in both groups: In polyomaviruses, transcription of early and late genes occurs in opposite polarity involving both strands. Papillomavirus DNA has only one transcribed strand with a long stretch of base pairs separating early and late transcripts. Polyomaviruses thus far appear to be oncogenic under experimental conditions only, mainly after injection into newborn animals. Papillomaviruses, in contrast, are the causative agents of papillomas and contribute, under certain conditions, to malignant conversion.

A. Polyomavirus Infection in Man

Two polyomavirus infections of man, BK and JC virus, were identified more than 10 years ago [2, 15]. BK virus is frequently excreted in the urine of immunosuppressed patients in high quantities without being identified as the causative agent of any human disease. JC virus was isolated from patients with progressive multifocal leukencephalopathy (PML) and appears to be the causative agent of this condition. Both viruses are oncogenic when inoculated into newborn rodents and transform hamster cells in tissue culture (reviewed in [20]). JC virus has also been shown to induce gliomas upon intracerebral inoculation into owl monkeys [12]. The occasional presence of SV40 antibodies in a small percentage of individuals (about 1%) and the identification of this virus in two cases of PML [21] may hint at the existence of human infections with this monkey virus.

Rather recently, evidence was obtained of human infections with an additional polyomavirus, the B-lymphotropic papovavirus (LPV). This virus was originally isolated from African Green Monkey (AGM) lymphoblasts [27]. Between 20% and 30% of the human adult population produce antibodies to this virus, which neutralize the AGM isolate and point to the existence of a closely related or identical agent in man. Attempts have failed thus far to demonstrate the pathogenicity of this virus or to correlate the antibody response to a specific disease pattern. Therefore, a possible role of polyomaviruses in human tumor induction remains to be elucidated.

B. Papillomavirus Infections in Man

Since 1976 [3, 5, 13], it has become apparent that several types and subtypes of papillomaviruses of man exist. So far, at least 17 distinct types of human papillomaviruses have been identified and it is predicted that additional types will follow.

Verrucae vulgares (common warts) is induced by human papillomavirus (HVP) types 1, 2, 4, and 7. The latter has only been found in butchers until now. Flat warts are caused by types 3 and 10, genital warts and laryngeal papillomas by types 6 and 11. At least seven distinct types have been isolated from patients with a rare skin disease, epidermodysplasia verruciformis (types 5, 8, 9, 12, 14, 15, and 17], and one virus isolate (type 13) originates from mucosal papillomas of the gingiva (morbus Heck).

C. Papillomaviruses and Human Cancer

More than 40 years ago, a papillomavirus infection of rabbits, the Shope papillomavirus, was identified as a potentially carcinogenic infection [16]. Additional application of chemical carcinogens at subthreshold concentrations enhances the malignant conversion and reduces significantly the latency period [17].

A few years ago, Jarrett and his colleagues [9] noted an interesting interaction between a papillomavirus infection and carcinogens in esophageal carcinomas of cattle. Papillomas caused by type 4 of bovine papillomavirus changed into squamous cell carcinomas in areas where cattle grazed on bracken, which contains a potent carcinogen.

A specific interaction between certain types of papillomavirus infections and physical and chemical carcinogens also seems to lead to certain human cancers (see review [24]); epidermodysplasia verruciformis represents a "classical" example. Generalized verrucosis is a pathognomonic feature of this condition. The papillomas are caused by distinct types of viruses. At least two types (types 5 and 8) have been identified in carcinomas arising within warts at a remarkable frequency [14], predominantly on sun-exposed sites of those patients. Skin carcinomas in these patients, therefore, seem to result from the interaction of a specific type of papillomavirus infection and the additional interaction with a physical carcinogen, namely the UV part of the sunlight.

A second example is provided by multifocal laryngeal papillomas, the majority of which seem to be caused by a predominantly genital papillomavirus (HPV-11) [6]. This condition reveals a remarkable tendency to recur after surgical removal and even iatrogenic spreading of the papillomas has been reported (reviewed in [24]). In previous decades, a number of attempts were made to treat these proliferations by x-irradiation. A high percentage of laryngeal papillomas treated under this regimen later converted into squamous cell carcinomas, even in young patients. Nonirradiated laryngeal papillomas very rarely convert into malignant tumors (see review [24]).

The most recent human condition to reveal increasing evidence for a similar interaction is human genital cancer. The epidemiology of this malignancy clearly points to an infectious event in its etiology (reviewed by [26]). Since 1968, herpes simplex viruses (HSV) have been suspected as playing a role in this condition. This was mainly based on seroepidemiologic studies pointing to a role of this virus in the etiology of genital cancer. Attempts to find HSV DNA in biopsy material derived from such tumors have largely failed, creating an obvious dilemma in linking HSV infections to genital cancer.

The failure to find HSV DNA in genital cancer biopsies stimulated the search for other viruses potentially involved in the etiology of this disease. In view of case reports on the malignant conversion of genital warts (see review [24]), human papillomaviruses appeared to be good candidates [23, 28, 29]. This led to the characterization of HPV-6 as the main agent found in genital warts (condylomata acuminata) by Gissmann and zur Hausen [4], the successful cloning of this virus in bacterial plasmids [1], and to the identification of a second agent, HPV-11, which prevails in preneoplastic dysplastic lesions of the cervix, but is also present in some condylomata acuminata [7].

The analysis of malignant genital tumors for HPV-6 or HPV-11 DNA has resulted in the following data: (1) Six of seven Buschke-Löwenstein tumors (giant condylomata acuminata or nonmetastasizing verrucous carcinomas) contained HPV- 6

or HPV-11 DNA; (2) 5 of 27 invasive carcinomas of the cervix or carcinomata in situ contained HPV-11 DNA or that of a related agent [7]. Since additional groups have reported as yet undefined papillomavirus DNAs in a few more tumors [8, 22] this clearly shows that at least a certain percentage of genital cancers (at present about 20%) contain papillomavirus DNA.

Very recently, our group cloned a third papillomavirus type directly from a cervical cancer biopsy (Dürst et al., unpublished data). This virus is very distantly related to HPV-6, HPV-11, or any of the other characterized human papillomaviruses. It will be of particular interest to test the as yet negative biopsies for the DNA of this new virus type, and such studies are presently in progress. These experiments are performed in the expectation that several distinct types of papillomaviruses cause infections of the human genital tract and a number of them may be involved in the malignant conversion of such papillomas.

We discussed previously the apparent interaction of papillomavirus infections with carcinogens (initiators) in the induction of malignant conversion. Is it possible to identify an initiator interacting with papillomavirus infections in the human genital tract? We believe this is indeed possible. This answer, at surprising first glance, refers to herpes simplex virus infections. Recently, we were able to demonstrate that HSV efficiently induces mutations within the hypoxanthin-guanine-phosphoribosyltransferase (HGPRT) locus of human rhabdomysarcoma cells [18]. More importantly, HSV was shown to share the property of inducing selective gene amplification [19] with chemical and physical carcinogens [10, 11]. This suggests that HSV may provide the initiating functions required for malignant conversion of papillomavirus "promoted" cells.

Very recent experiment (Gissmann et al. unpublished data) provide direct evidence for the amplification of papillomavirus sequences in HSV-infected cells, stressing the probability of the suggested interaction.

The available data have led to the development of a model [25] claiming that human genital cancer results from a synergistic interaction between two virus infections (papillomavirus and HSV) or one virus infection (papillomavirus) and other initiating events.

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Characterization of Proteins Induced by *Herpesvirus saimiri*: Comparative Immunoprecipitation and Analysis of Glycosylation

S. Modrow, H. Schmidt, and H. Wolf

Marmosets of the genus *Saguinus* (*S. nigricollis, S. fuscicollis,* and *S. oedipus*) are highly susceptible to tumor induction by *Herpesvirus saimiri* and die from a rapidly growing neoplastic disease [1–3] following viral infection. Owl monkeys (*Aotus trivirgatus*) show a similar course of disease. The appearance of tumors in these animal is often delayed and about 20% of the owl monkeys do not develop tumors [4, 5]. *Herpesvirus saimiri*-infected New Zealand White Rabbits (NZWR) show a disease pattern similar to the primate tumors; the incidence of neoplastic disease after infection with *H. saimiri* ranges from 20% to 75% in different studies [6–8]. The specificity of antibodies in the various experimental and natural hosts (*Saimiri scireus*) was determined by immunoprecipitation of viral polypeptides obtained from owl monkey kidney (OMK) cells infected with *H. saimiri* in the presence of labeled precursors. Whereas we describe the unglycosylated virus-induced proteins involved in another report [9], we include in this report the description of the glycoproteins in *H. saimiri*-infected cells.

In this study, we infected OMK cells with *H. saimiri* (strain 11) and an attenuated mutant of *H. saimiri,* originally isolated by Schaffer [10]. At various times (6–8 h, 15–17 h, and 24–26 h after infection), virus-induced cell proteins were labeled with ^{35}S-methionine (20 μCi/ml) and the viral proteins were immunoprecipitated with various sera from infected animals (the sera from the natural host *Saimiri sciureus* and from infected owl monkeys were a gift from L. Falk, New England Primate Center). A class of early proteins was obtained from infected cells by treatment with azetidine (Sigma); they were precipitated with the same sera.

The protein profiles obtained after precipitation with sera from *H. saimiri*-infected experimental hosts (owl monkeys, white lip marmoset, and NZWR) differed from those obtained with sera from the natural hosts. Proteins precipitated with sera from *Saimiri sciureus* were mostly late proteins and components of the virion. Sera from the experimental hosts precipitated a limited number of proteins (p 152, p 127, p 115, p 80, p 55–57, p 53, and p 50). Three of them (p 115, p 80, and p 55–57) were not found with sera of the natural hosts. p 115 and p 55–57 are already synthesized at an early stage after infection; the synthesis of p 115, however, is inhibited by treatment with azetidine and thus may belong to a second group of early proteins. p 127 and p 152 are components of the viral capsid. Natural and experimental hosts of *H. saimiri* revealed distinct profiles, whereas some similarities existed among the various types of experimental hosts. The reason for the observed differences may be the expression of viral polypeptides in the various host cells or host-specific differences in the immune system. No difference could be detected between the patterns obtained with the oncogenic *H. saimiri* 11 and the attenuated strain (*H. saimiri* 11 att.).

In a second line of experiments, we characterized glycosylated proteins produced in *H. saimiri*-infected OMK cells. Two different methods were used: (1) in vivo labeling with ^{14}C-glucosamine in a medium containing fructose instead of glu-

cose and followed by immunoprecipitation; (2) in vitro labeling of the glycoproteins separated on SDS-polyacrylamide gels with ^{125}I-labeled lectins. Both experiments showed a similar pattern of glycosylated viral proteins produced in infected cells and can be used interchangeably. Seven glycosylated proteins could be identified (p 152, p 140, p 127, p 88, p 67, p 53, p 50) with both methods.

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Structural Organization and Expression of the Epstein-Barr Virus Genome*

G. W. Bornkamm, U. K. Freese, G. Laux, J. Hudewentz, and H. Delius

Infection of B-lymphocytes with Epstein-Barr virus (EBV) induces unlimited growth of the cells in culture. Cells immortalized by EBV harbor the viral genome and express the virus-specific nuclear antigen EBNA.

In EBV-carrying cell lines usually a small and varying percentage of cells is spontaneously induced to produce viral particle (producer lines). Treatment of the cells with the tumor promoter 12-0-tetradecanoyl-phorbol-13-acetate (TPA) and a number of other inducers increases the number of virus-producing cells. In some cell lines TPA induces only the synthesis of early viral antigens (nonproducer lines) [6].

The EBV genome isolated from purified virus particles is a linear double-stranded DNA molecule of 180,000 bp, carrying identical repeats of about 400 bp at both termini. It is composed of a short unique region (U_S) of 15 kbp and a long unique region (U_L) of 130 kbp, separated from each other by an array of 3.1-kpb repeats in tandem orientation. The number of repeats varies between 6 and 12 among different virus isolates and is variable even within one DNA population.

Sequences located in *Bam HI-H* at the left hand side of the long unique region have been shown to share some sequence homology with sequences about 105 kbp apart in *Bam HI-B1* at the right hand side of the long unique region [4]. The regions with sequence homologies are denoted DS_L and DS_R (left and right duplicated sequence). DS_R is located within the 12 kbp, which are delated in the B 95-8 strain of EBV.

The observation that DS_L and DS_R are actively transcribed after treatment of the cells with TPA led us to a more detailed analysis of the two regions with sequence homologies and their transcription products.

Recombinant plasmids containing DS_L and DS_R were obtained by cloning the DNA of M-ABA (EBV), a virus, originally derived from a nasopharyngeal carcinoma, which was shown to have the genomic organization of the EBV prototype [1]. Cleavage of the DNA of a clone containing DS_R with a number of different restriction endonucleases revealed a so far unrecognized small tandem repeat of 102 bp. Heteroduplexes of the DNA of the two clones containing DS_L and DS_R, respectively, visualized in the electron microscope by cytochrome c spreading, revealed that the region of homology is about 2.5 kbp long, involves the small repeats, and has the same orientation in the viral genome. Mica adsorption of the heteroduplex without cytochrome c showed that the region with sequence homology consists of about 1.5 kbp with partial homology including the small repeats and 0.9 kbp with well-matched duplexes. The involvement of the DS_R repeats in the heteroduplex formation suggested the presence of repeats also in DS_L. Reannealing of DNA containing the DS_L region can give rise to the formation of two loops of the same size, which is expected for a DNA containing tandem sequence reiterations. The presence of repeats in DS_L,

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including a restriction site for *NotI*, has recently been reported [2].

In the nonproducer cell line Raji transcription of DS_L and DS_R is switched on after TPA treatment. For the analysis of viral transcripts, cytoplasmic RNA was isolated from Raji and M-ABA cells treated with and without TPA and was run in formaldehyde agarose gels. The RNA was transferred from the gel to nitrocellulose according to Thomas [5]. Hybridization with ^{32}P-labeled cloned viral DNA fragments revealed that the RNA species coded for by DS_L and DS_R are 2.8 and 2.6 kb long. They are the most abundant transcripts after TPA induction. Hybridization with subclones showed that both RNA species are almost exclusively transcribed from the DS_L and DS_R repeats, respectively. Both RNA species are polyadenylated. Sequencing of the DS_R repeat revealed six open reading frames.

Whether the RNAs coded for by DS_L and DS_R are translated into polypeptides with a presumably repetitive amino acid sequence remains to be elucidated.

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Induction of Latent Epstein-Barr Virus Information by a Serum Factor

G. Bauer and H. zur Hausen

Epstein-Barr virus (EBV) is known as the causative agent of infectious mononucleosis and is possibly associated with the development of Burkitt's lymphoma and nasopharyngeal carcinoma. Latently infected lymphoblastoid cell lines can be obtained from patients with EBV-associated diseases, seropositive donors, and by in vitro immortalization of cord blood lymphocytes with certain strains of EBV. Cell lines containing persisting EBV DNA are commonly used to study the mechanisms that regulate latency of EBV.

The expression of viral antigens in EBV genome positive lymphoblastoid cell lines is controlled by so far undefined viral or cellular regulatory mechanisms. In nonproducer lines, only Epstein-Barr virus nuclear antigen (EBNA) is expressed in virtually all cells within the population, whereas the synthesis of viral early antigens (EA) and viral capsid antigens (VCA) is blocked. The latent information may be induced to give rise to the synthesis of viral antigens by various treatments, such as culturing in arginine-deficient medium, addition of inhibitors of protein synthesis, halogenated pyrimidine analogues, antibody to human IgM, tumor-promoting phorbolesters (TPA), *n*-butyric acid, and in some lines with 5-azacytidine and intercalating agents.

Here we summarize the characterization of a humoral protein that can induce the synthesis of viral antigens and that cooperates with chemical inducers in the induction process.

A. Inducing and Cooperative Serum Factor

I. Effects

Serum factor induces the synthesis of viral antigens in latently infected cells. The kinetics of induction resembles those obtained by chemical inducers.

Serum factor cooperates with chemically different inducers such as TPA, *n*-butyric acid, anti-IgM, and IUdR in the induction process. Cooperation is characterized by a total induction that is much higher than the sum of inductions reached by individual inducers, and by a substantial shift of the dose-response relationship between chemical inducer and induction into a more sensitive range.

II. Nature of the Inducing Serum Factor

Inducing serum factor has been purified from calf serum (300,000-fold enrichment). It is a relatively heat-stable protein of about 500,000 daltons. Nanograms per millimeter of the purified material are sufficient for measurable effects. The factor is composed of subunits.

III. Activation of the Serum Factor

Ninety-five percent of serum factor is present in inactive form. Activation in vitro is carried out by treatment with alkali and acid. The activation reaction has been classified as a conformational change within the molecule. The mechanism of activation of the molecule in vivo is presently only known to exist but is not characterized.

IV. Occurrence

Inducing serum factor has been demonstrated in the sera of all vertebrates.

V. Specificity for Defined Cell Lines

Only several Burkitt's lymphoma cell lines and marmoset lines can be induced by the factor. Lymphoblastoid cell lines from seropositive donors or lines established by in vitro immortalization of human cord blood lymphocytes fail to show any response to the factor. The factor therefore seems to recognize cellular markers associated with the state of differentiation.

VI. Inhibition

The action of serum factor can be inhibited by retinoic acid, in analogy to the induction by tumor promoters and by other drugs.

B. Conclusions

The factor described here may play a central role in the regulation of EBV gene activity by virtue of both its inducing capacity and its dramatic enhancement of the action of inducing drugs. Thus it may have a central role in processes that overcome latency of EBV.

The widespread occurrence and the conserved nature of the molecule point to a more general physiological function. EBV induction may be an epiphenomenon of this hypothetical process. The existence of inactive and active forms of the factor indicates the possibility of modulation of total factor activity by an activating mechanism. The interaction of factor with chemically different inducers points to the action of the molecule at a central step in the molecular events during induction. Inhibition by retinoic acid points to the utilization of at least one common pathway in the course of induction of viral information and tumor promotion.

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Thymic Nurse Cells and Radiation Leukemia Virus Induced Thymic Lymphomas in C57BL Mice*

J. Boniver and M. P. Houben-Defresne

A. Introduction

The selective thymotropism of *radiation leukemia virus* is an interesting example of specific interaction between an oncogenic agent and the differentiation pathway of a well-defined cell lineage. Indeed, the inoculation of RadLV in C57BL/Ka mice leads to the development of thymic lymphomas [4].

Only a few immature thymocytes can act as target cells for productive infection by RadLV [1]. Infection is followed by virus replication which starts in the outer cortex to spread rapidly to the whole cortical thymocyte population [2]. Thymus-dependent preleukemic cells emerge during the first phase of virus replication, although the thymus is still not tumoral. Finally thymus-independent neoplastic cells appear and give rise to lymphomas (Boniver et al., to be published).

The immaturity of target cells and the thymus dependency of the preleukemic process led us to investigate the possible role of *thymic nurse cells* (TNCs) in thymic lymphomagenesis. These lymphoepithelial cell complexes are indeed at a critical stage of the early thymic lymphopoiesis [3, 5, 6].

B. Results

I. TNCs and Virus Replication

Early after the intrathymic inoculation of RadLV, the first virus-producing cells were found preferentially within TNCs: on day 2, their frequency was 200 times higher in the TNC-associated lymphoid cells than in the whole thymocyte population [3]. Later on, virus replication spread to the whole cortex [1, 2].

II. TNCs and Preleukemic Cells

Thymus-dependent preleukemic cells were detected on day 2 after inoculation of RadLV using an in vivo transplantation test. Thymus-independent preleukemic cells, i.e., able to proliferate into thymectomized mice, were found in non-tumoral thymuses between the 75th and the 120th day (Boniver et al., to be published).

The relation of preleukemic cells to TNCs was then investigated. C57BL/Ka (Thy 1.2) mice were killed at various time intervals after inoculation of RadLV. In order to obtain TNCs, thymuses were dissociated with enzymes (S start) and separated in several fractions (S1 . . . S4, TNCs) by 1-g sedimentation [3]. Only S start and TNCs fraction contained TNCs. Samples of each cell suspension obtained with this procedure were injected intrathymically into 400-R irradiated congenic C57BL/Ka (Thy 1.1) mice, which were killed when moribund. The donor, or recipient, origin of lymphomas was scored by Thy 1.1 and Thy 1.2 detection with a FACS. In this transplantation assay, only the donor type tumors indicated the presence of preleukemic cells in the inoculate. Recipient-type tumors were due to transfer of infectious virus particles.

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Table 1. TNCs and preleukemic cells after inoculation of RadLV

Time of killing[a] (days)	Thymocyte number[b] ($\times 10^6$)	TNC number/ thymus[c] ($\times 10^3$)	Preleukemic cells in thymocyte fractions obtained along the TNC isolation procedure[d]					
			S start	S1	S2	S3	S4	TNCs
2	81	13.4	+	–	–	–	–	+
15	97	3.7	–	–	–	–	–	+
30	93	2.1	+	+	+	+	ND[e]	+
45	94	2.3	+	–	+	ND	ND	+
60	113	0.56	+	–	+	ND	ND	+

[a] C57BL/Ka were inoculated with RadLV at 30 days of age
[b] Normal values: 30 days old, 170.10^6; 90 days old, 125.10^6
[c] Normal values: 30 days old, 15.10^3; 90 days old, 10.10^3
[d] Tested by in vivo transplantation assay (see text)
[e] ND; no cells in the fraction

As shown in Table 1, preleukemic cells were found only in TNCs on day 2 and 15. Later on preleukemic cells were detected also in cell suspensions which did not contain TNCs (i.e., S1 to S3). Interestingly the number of TNCs per thymus dropped drastically from day 15 onward to reach very low values on day 60, whereas it decreased very slowly in control mice.

C. Discussion

The intra-TNC localization of early virus replication after inoculation of RadLV can be explained easily: both target cells for productive infection by RadLV and intra-TNC lymphoid cells display an "immature" phenotype and therefore probably belong to an unique subpopulation at the first stage of thymic lymphopoiesis. The first preleukemic cells might derive from the same population. Their selective association with TNCs, for about 2 weeks, suggests that the RadLV-induced preleukemic potential is restricted to a specific (intra-TNC) step of T-cell differentiation. The almost complete disappearance of TNCs and the spread of preleukemic cells to various thymocyte fractions indicate a blockade of the physiological interactions which normally maintain lymphopoiesis.

The observations strongly indicate that the selective thymotropism of RadLV relates to specific interactions with well-defined susceptible target cells; these target cells are at a critical stage of the T-cell differentiation pathway, under control of the thymic microenvironment, and particularly of the epithelial component of TNCs.

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Construction and Characterization of Chromosomal DNA Libraries*

B. D. Young, M. Jeanpierre, M. H. Goyns, G. D. Stewart, T. Elliot, and R. Krumlauf

A. Summary

The construction of an extensive human genetic linkage map will require the generation of large numbers of DNA probes specific for single chromosomes. Recombinant DNA libraries representing chromosomes 22 and 21 have been constructed with a view to studying the specific rearrangements of chromosome 22 observed in chronic myeloid leukaemia and Burkitt's lymphoma and also the association of chromosome 21 with Down's syndrome. This was accomplished by sorting about 2×10^6 copies of chromosome 22 and 21 by flow cytometry and insertion of the DNA into the vector *λgt WESλB*. Twenty clones selected at random from the chromosome 22 library hybridised to *Eco*RI-digested human DNA, and five of these hybridised to single bands identical in size to the phage inserts. Altogether six single-copy sequences and a clone coding for an 8S RNA isolated by screening the chromosome 22 library for expressed sequences were characterised in detail. Hybridisation of all seven clones to a panel of sorted chromosomes and hybrid cell lines confirmed the assignment of the sequences to chromosome 22. The sequences were localised to regions of chromosome 22 by hybridisation to translocated chromosomes sorted from a cell line having a balanced translocation *t(17;22)(p13;q11)* and to hybrid cell lines containing the various portions of another translocation *t(X;22)(q13;q112)*. Five clones reside on the long arm of chromosome 22 between *q112* and *qter*, while two clones and an 18S rRNA gene isolated from the chromosome 22 library reside between *pter* and *q112*. In situ hybridisation has further mapped one of these clones to the region *q13-qter*. An identical approach was used for chromosome 21 and three out of five single copy clones have been identified as specific to this chromosome by hybridisation to DNA from a cell hybrid containing only human chromosome 21. In summary, it has been shown that this approach has yielded DNA libraries of high purity based on chromosomes 21 and 22. This method has the advantage of being direct and applicable to nearly all human chromosomes and will be important in the molecular analyses of human genetic disease.

B. Introduction

The availability of cloned genes has rapidly expanded our knowledge of the fine structure, organisation and expression of sequences in the human genome, and provided insight into the molecular basis of diseases such as the thalassemia syndromes. In addition, the application of recombinant DNA technology to human genetics has played an important role in attempting to bridge the gap in resolving power between DNA sequence analysis and classical cytological approaches to mapping and gene linkage. Recent studies have shown that highly polymorphic loci can be identified from human genomic libraries (see review [1]). Using such probes the construction of

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a fine structure linkage map would be particularly useful in cases where the chromosomal location of the genetic lesion associated with a disease is known. In this context a general method for rapidly isolating a wide spectrum of DNA probes for specific human chromosomes would be an invaluable aid to human gene mapping. An essential prerequisite for such an approach is the high resolution fractionation of human chromosomes.

There have been many attempts to fractionate metaphase chromosomes using centrifugation [2], counter-current distribution [3], and 1 g sedimentation [4]. While such attempts have achieved some enrichment of different size classes of chromosomes, the similar size of many human chromosomes has prevented the separation of pure fractions of single chromosomes. The construction of human-rodent cell hybrids has allowed the purification of single human chromosomes using selectable markers [5]. Although this approach has the disadvantage of the rodent cell DNA background, it has been used to obtain clones from particular chromosomes [6]. However, due to the presence of a larger number of clones containing rodent cell DNA, this approach is useful for obtaining only small number of clones. In order to obtain a larger number of clones from a particular chromosome, it is necessary to purify the chromosomes of interest in sufficient quantity to allow direct DNA cloning. We have already reported the sorting of the human chromosomes 22 and 21 by flow cytometry in sufficient quantity to allow the construction of DNA libraries [7]. In this report we extend these observations to include the mapping of more clones on both chromosomes 22 and 21. Chromosomes 22 and 21 were chosen for this study in view of the specific rearrangements of 22 which occur in chronic myeloid leukaemia and the association of 21 with Down's syndrome.

C. Results

I. Sorting and Cloning of Chromosomes 21 and 22

The strategy we have adopted for obtaining DNA sequences from human chromosomes 21 and 22 involves directly sorting these chromosomes on a fluorescence-activated cell sorter (FACS II). A high-resolution flow analysis of a suspension of metaphase chromosomes prepared [8] from the lym-

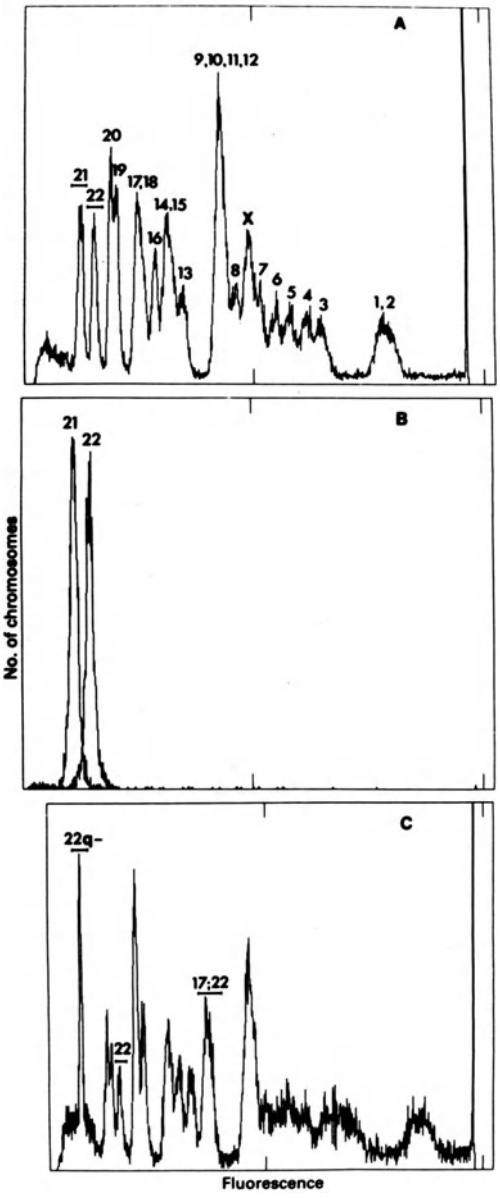

Fig. 1A–C. Flow karyotypes of human chromosomes. *Numbers* indicate the chromosomes contained in each peak, and the *horizontal bars* indicate the fluorescent windows used to sort chromosomes. **A** GM1416: 48,XXXX lymphoblastoid cells. **B** Fractions of chromosomes 21 and 22 sorted from **A**. **C** GM3197: 46,XX cells bearing the translocation *t(17;22)(p13;q11)*

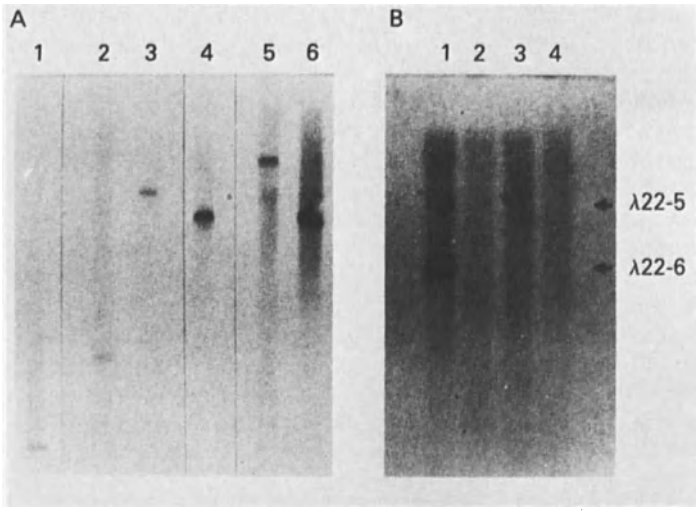

Fig. 2A, B. **A** Hybridisation of single-copy clones. Nitrocellulose strips containing 20 μg of GM1416 DNA digested with *Eco*RI were hybridised with probes prepared from various recombinant phages. *Lanes: 1, λ22-1; 2, λ22-2; 3, λ22-3; 4, λ22-4E; 5, λ22-5; 6, λ22-6.* **B** Assignment of probes to chromosome 22. Clones *λ22-5* and *λ22-6* were labelled separately, mixed, and hybridised to *Eco*RI-digested DNA from sorted fractions containing 1×10^6 copies of various chromosomes. *Lanes: 1,* chromosome 22; *2,* chromosome 21; *3,* chromosomes 1–8; *4,* chromosomes 9–20

phoblast cell line GM 1416: 48, XXXX, generates the flow karyotype shown in Fig. 1A. The assignment of chromosomes to each of the distinct peaks was based on their DNA contents [9] and on the results of a study which established correlations between flow karyotypes and conventional G- and C-banding karyotypes using abnormal human chromosomes and heterochromatic polymorphisms [10]. The peaks containing chromosomes 21 and 22 were individually sorted, and a sample of each was reanalysed on the FACS II to assess the efficiency of sorting (Fig. 1 B). Each fluorescence profile showed a sharp single peak, suggesting the sorted fractions were pure.

DNA was extracted from each fraction containing 2×10^6 chromosomes (about 90–100 ng DNA) and was used to construct recombinant libraries in *λgt WES λB* by a modification of the method described by Maniatis et al. [11]. The DNA was completely digested with *Eco*RI, due to the difficulty in controlling digestion of such small amounts. A yield of 3×10^5 unamplified recombinant phage was obtained for each chromosome, which should be sufficient to ensure that the majority of sequences were represented in the libraries. The inserts of all phage examined to date range in size between 2.5 and 14 kilobase pairs. A background of parental phage (about 10%) was estimated by ligation and packaging of cloning arms alone.

II. Isolation and Assignment of Single-Copy Sequences to Chromosome 22

Single-copy sequences required for linkage analysis were initially identified by isolating phage DNA from 20 recombinants selected at random. The DNAs were labelled with P^{32} by nick translation [12] and hybridised to nitrocellulose strips containing *Eco*RI-digested total DNA from GM 1416 cells. All the probes hybridised to the filters, indicating that they contained cloned human sequences. The hybridisation from 13 probes produced smears, indicating that they contained sequences repeated with varying frequencies in the human genome [13]. In two cases, complex multiple bands were observed. However, the remaining clones hybridised to discrete DNA fragments identical in size with their human DNA inserts (Fig. 2A). This verifies that

the sorting and cloning procedures have not altered the chromosomal DNA. Based on these hybridisation data, we initially selected these five recombinants as single-copy probes for further characterisation and mapping. The assignment of these sequences to chromosome 22 was determined by hybridisation to DNA from sorted chromosomes. Results for two of the clones are shown in Fig. 2B. DNA from *λ22-5* and *λ22-6* was labelled separately by nick translation, mixed and hybridised to a filter containing DNA from four sorted fractions corresponding to chromosomes 22, 21, 1–8 and 9–20. Both probes hybridise to fragments of the appropriate size only in the fraction containing chromosome 22 (Fig. 2B). This illustrated the high degree of purity of the sorted chromosomes, and allowed us to assign the five clones to chromosome 22. The presence of these sequences on the chromosome has been independently confirmed by hybridisation to a series of hybrid cell lines. The probes hybridise to only those lines containing human chromosome 22 (data not shown).

III. Localisation of Clones on Chromosome 22

We have utilised three approaches to localise the cloned sequences to specific regions of chromosome 22, based on methods used to map globin genes [14] and non-coding sequences on chromosome 11 [6]. First, we have sorted the two respective portions of one homologue of chromosome 22 from the translocated cell line GM 3197: 46, XX, *t(17;22)(p13;q11)*. The flow karyotype of this line showed that the balanced translocation produced derivative chromosomes that were sufficiently different in size from the normal homologue that they could be identified and sorted on the FACS II (Fig. 1C). By hybridising each of the probes to Southern blots of DNA from the two sorted portions of chromosome 22, the sequences were localised to a region of the chromosome. For example, clone *λ22-1* hybridises only to total DNA and the *22q-* derivative placing it between *pter-q11* (Fig. 3A); while clones *λ22-5* and *λ22-6* are detected only in the total and 17:22 derivative fractions, mapping them to *q11-qter* (Fig. 3B).

In a similar manner, we have used DNA from several human-hamster cell lines derived from the fibroblasts of a human female carrying a *t(X;22)(q13;q112)* reciprocal translocation [14]. The hybrid lines contain either a normal homologue of human chromosome 22 or a segregant of one of the two derivative chromosomes (*X/22, 22/X*) free of its normal counterpart. Probes *λ22-5* and *λ22-6* hybridised to the lane containing *X/22* DNA not *22/X*, placing them between *q112-qter* (Fig. 4A, B). Clone *λ22-1* again showed the reverse pattern, localising it to the *pter-q112* region (Fig. 4C). Since the breakpoints of the *X;22* and *17;22* translocations mapped in the same general region of chromosome 22 (*q112* vs *q11*), we expected the results to be similar. They were identical for each of the five probes, confirming the map assignments made using the sorted translocations. In situ hybridisation [15] was also used to map clone *λ22-5* on chromosome 22. The hybridisation of this clone to normal human chromosomes is presented in Fig. 5A as a histogram of grain count versus chromosome number and it can be seen that there is a distinct peak over chromosome 22. This clone was also hybridised against the chromosomes of a cell line which bore the translocation *t(11;22)(q25;q13)* and it can be seen in Fig. 5B that there is a peak of hybridisation over both the normal chromosome 22 and over the chromosome 11/22 but not over 22/11. Thus it can be concluded that clone *λ22-5* lies distal to the breakpoint in *q13* on chromosome 22. Figure 7 summarises the results and relative map positions of these clones on chromosome 22.

IV. Expressed Sequences

We have screened the chromosome 22 library for coding sequences. A cDNA prepared from polyadenylated RNA isolated from the white cells of a patient with chronic myeloid leukaemia was used to probe an aliquot of the phage. Recombinants giving strong positive signals with this probe were picked for further analysis. A Northern blot of one of these clones (*λ22-4E*) against polyadenylated RNA from several types of human cells shows that it is expressed in many cell types, and suggests that it codes

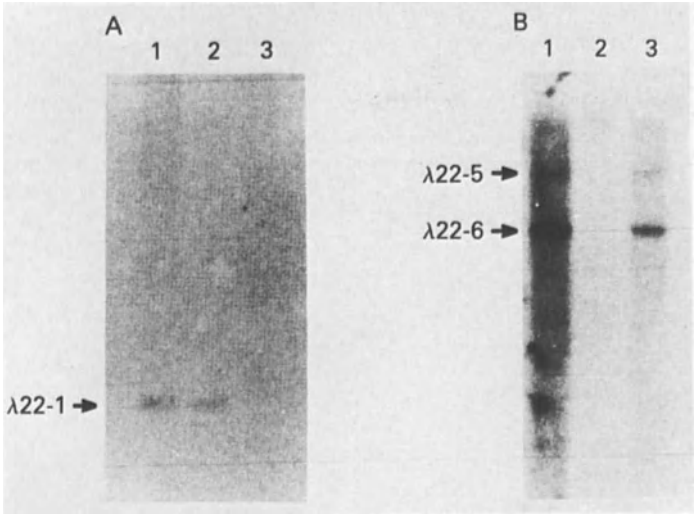

Fig. 3A, B. Mapping probes with sorted translocations. Cell line GM3197 bearing the translocation *t(17;22)(p13;q11)* was used for sorting 7×10^5 copies of the two portions [*22q-(lane 2)* and *17; 22(lane 3)*] involved in the translocation, and the normal homologue of chromosome 22 (*lane 1*) as indicated in Fig. 10. The filter containing *Eco*RI-digested DNA from these fractions was first hybridised with labelled phage DNA from clone *22-1* **A,** then melted and rehybridised with mixed probes from clones *λ22-5* and *λ22-6* **B**

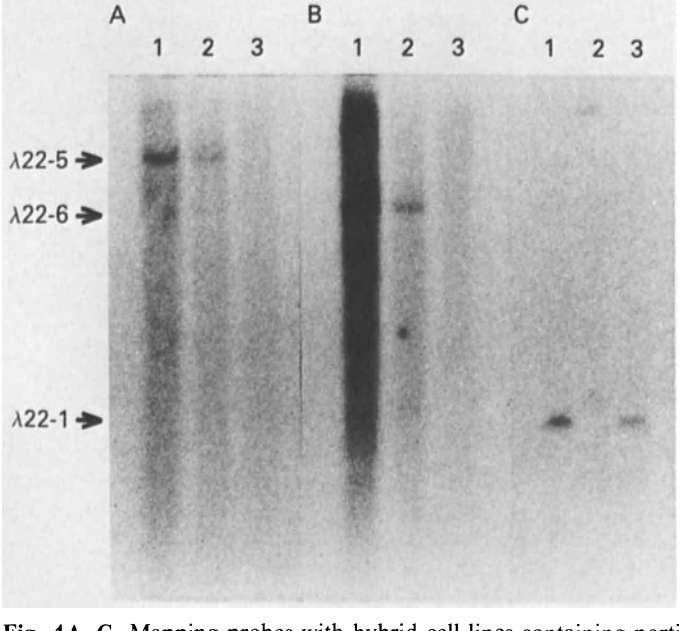

Fig. 4A–C. Mapping probes with hybrid cell lines containing portions of chromosome 22 resulting from the translocation *t(X;22)(p13;q112).* Filters containing *Eco*RI digests (20 μg/lane) of human DNA (*lane 1*), and DNA from hybrid lines S and H (*lanes 2 and 3* respectively) carrying the *X/22* and *22/X* portions of the translocation were hybridised to the nick translated phage *λ22-5* **A,** *λ22-6* **B** and *λ22-1* **C**

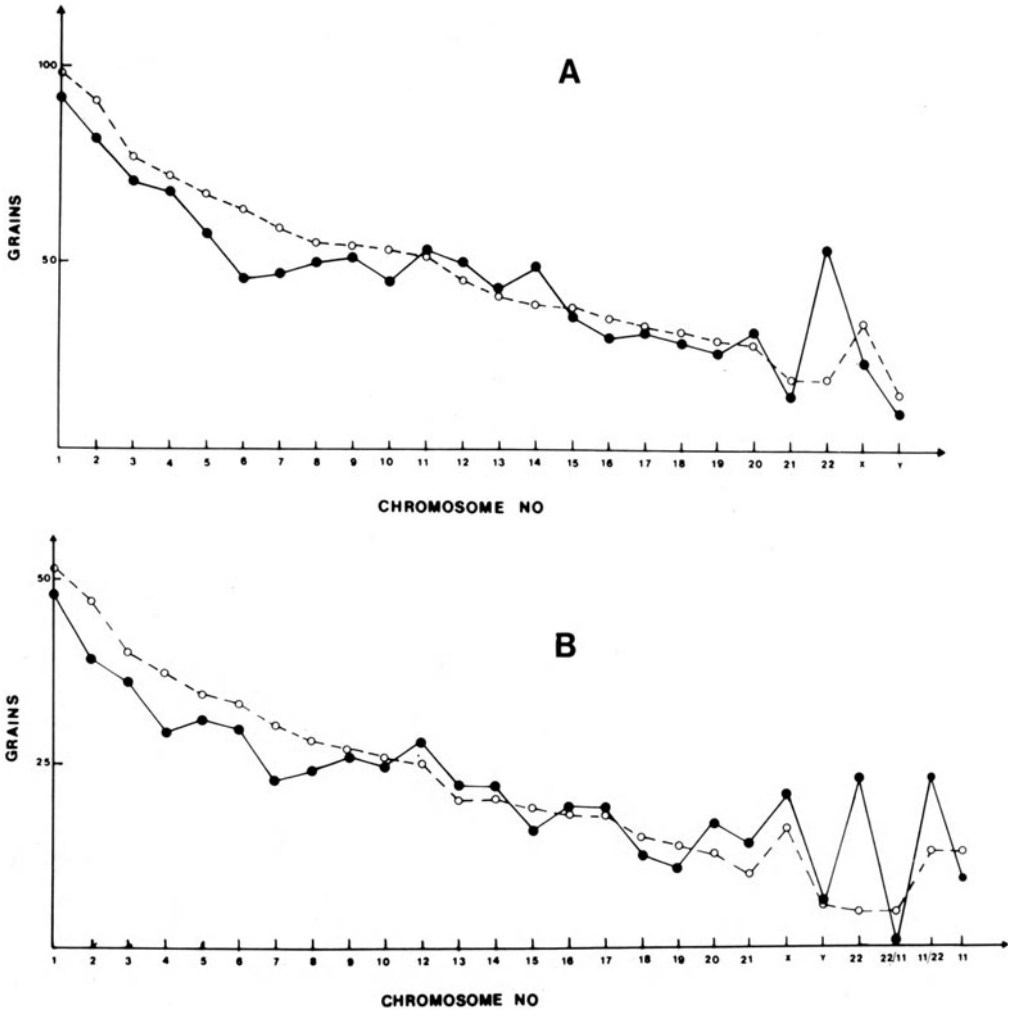

Fig. 5 A, B. Results of in situ hybridisation of nick translated *λ22-5* phage DNA (10^7 dpm/μg) to metaphase chromosomes of normal lymphocytes **A** and to those of a lymphoblastoid cell line **B** bearing the translocation *t(11;22)(q25;q13).* Grains were counted over 39 normal metaphase lymphocytes **A** and over 15 translocation-bearing lymphocytes **B.** The *solid line* represents actual grain counts and the *broken line* represents the expected values if the grains had been randomly distributed according to chromosome length

for an 8S mRNA (Fig. 6 B). The probe hybridises to a variety of fragments in total human DNA, besides the one corresponding to its insert, suggesting that is may be encoded by multiple genes. Using DNA from the X/22 somatic hybrid lines we have mapped the sequence corresponding to the *λ22-4E* insert to the *q112-qter* region of chromomosome 22 (Fig. 6 A). Finally, chromosome 22 has a nucleolar organiser and we have identified several 18S and 28S rRNA clones using a ribosomal probe. One clone in particular, 22-18S, has been characterised and found to contain a 6.6-kilobase pair fragment from the human ribosomal repeat unit coding for the 18S rRNA (data not shown).

V. Localisation of Clones on Chromosome 21

In a manner similar to that described above for the chromosome 22 library, 96 clones

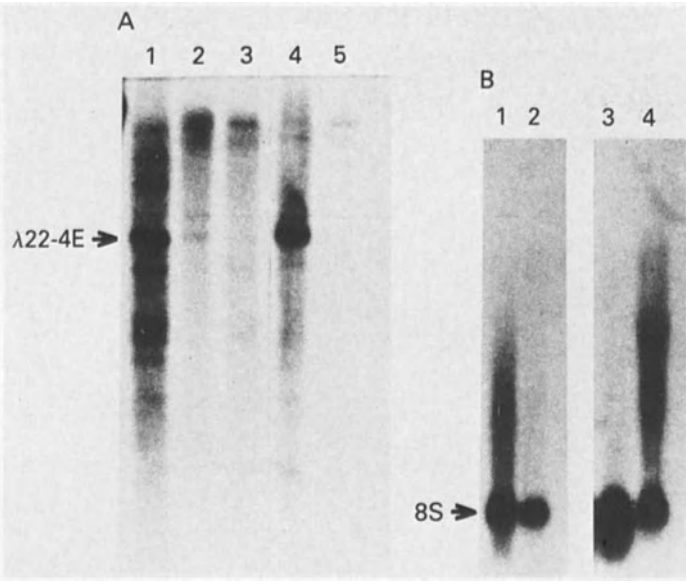

Fig. 6A, B. Characterisation of an expressed clone. **A** Clone *λ22-4E* was mapped by hybridisation to a filter with *Eco*RI digests (20 μg/lane) of human DNA (*lane 1*), Chinese hamster DNA (*lane 5*) and DNA from the hybrid cell lines M, H and S containing various portions of chromosome 22 [*22, 22/X* (*lane 2*), *22/X* (*lane 3*), *X/22* (*lane 4*)]. **B** The labelled phage, *λ22-4E,* was also hybridised to a filter containing 3 μg of total polyadenylated RNA isolated from a patient with chronic granulocytic leukaemia (*lane 1*), a patient with chronic lymphatic leukaemia (*lane 2*), cell line K562 (*lane 3*) and the MRC-5 human fibroblast line (*lane 4*)

selected at random were screened with nick translated human DNA. Twenty clones did not hybridise, indicating that they may have had single copy inserts. Six of these twenty clones were found to have single copy inserts and hybridised to *Eco*RI-digested human and mouse DNA and also to DNA from a rodent-human cell hybrid which contained only chromosome 21. As shown in Fig. 7, three of these clones (B3, H8 and D4) hybridised to the cell hybrid DNA, thus localising them to chromosome 21. Of the other three clones, two do not appear to be on chromosome 21 (A6 and B4) and one apparently does not contain human DNA.

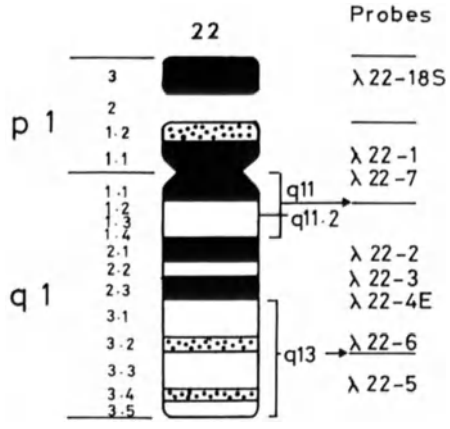

Fig. 7. Summary of mapping results on chromosome 22. Schematic representation of chromosome 22 showing the breakpoint positions of the *X/22 (q112), 17/22 (q11)* and *11/22 (q13)* translocations used for mapping as described in the text. The relative positions of the clones are indicated.

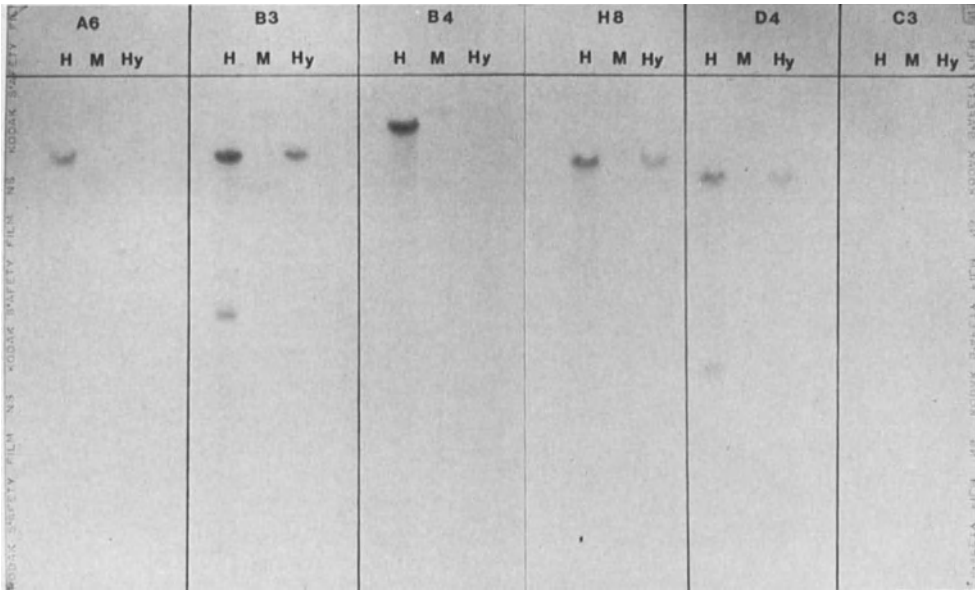

Fig. 8. Mapping probes from chromosome 21 library with a hybrid cell line containing only chromosome 21. Filters containing *Eco*RI digests of human DNA (*H*), mouse parent cell line BW5147 (*M*) and the hybrid cell line Thy B133R (*Hy*) were hybridised to six different nick translated phages

D. Discussion

This study on chromosomes 21 and 22 shows that by using the fluorescence-activated cell sorter to isolate purified fractions of specific human metaphase chromosomes, it is possible to construct DNA libraries representing single human chromosomes. The construction of a chromosome 22 library as a total *Eco*RI digest has enabled us to rapidly isolate and localise single-copy sequences on the chromosome. Analysis of the chromosome 22 library so far indicates that the background of parental phage is low, since 50 recombinants selected at random have all been found to contain human DNA inserts. Of these, ten single-copy clones have been identified and nine have been shown to be on chromosome 22 and one on chromosome 21. The map positions of eight of these sequences are summarised in Fig. 7. This illustrates the purity of chromosomes obtained with the sorting procedure, and suggests that the majority of sequences in this library are derived from chromosome 22.

This approach is simple and direct, offering several advantages over methods utilising total genomic libraries [16] or libraries prepared from human-rodent hybrid cell lines [6]. Relatively small numbers of phage are usually handled, since prescreening steps to identify human clones among a rodent background are not necessary. The level of purity of the sorted chromosomes ensures that most of the clones are derived from the same chromosome. Repetitive sequences can be isolated and characterised regardless of their reiteration frequency or cross-species hybridisation. Sequences on chromosomes that exhibit hybrid instability or lack selectable enzyme markers may also be isolated, since hybrid cell lines do not need to be constructed. Furthermore, since this library consists of sequences representing only 1.5% of the genome, relatively little phage (10,000) needs to be screened to isolate genes located on the chromosome. Of particular interest are the λ-immunoglobulin light chain genes, located on chromosome 22 [17], which are possibly involved in the translocation between chromosomes 8 and 22 in a variant form of Burkitt's lymphoma [18]. A second 22 library prepared following complete digestion with *Bam*HI will allow us to chromosome walk from any sequences of interest identified from the first library.

The application of our method to all of the human chromosomes should be possible. We have sorted an X chromosome fraction from the human 4X cell line GM 1416, which Davies et al. [19] have also used to construct a recombinant library and characterise several X-specific clones. An essentially identical approach has been used by Kunkel et al. [20] to obtain cloned DNA segments of the human X chromosome and by Disteche et al. [21] to obtain cloned DNA segments of the mouse X chromosome. Further successful extensions of this technique depend upon the sorting and resolution of chromosomes by flow cytometry. A typical flow karyotype can resolve human chromosomes into about 17 distinct peaks that can be sorted, 13 of which correspond to single human chromosomes (Fig. 1A). The combined use of different types of fluorescent dyes can also provide further separation of some groups of chromosomes [22]. Many of the remaining chromosomes, for example, chromosomes 1, 2 and 9, are separable from each other on the basis of shifts that have been observed in the fluorescence profiles of chromosomes of similar size, resulting from abnormalities and heterochromatic polymorphisms [10]. It is particularly important that selected specific regions of some chromosomes can also be sorted and cloned using cell lines containing deletions and other derivative chromosomes from rearrangements.

The combination of chromosome sorting and recombinant DNA techniques offers the opportunity for investigating several important areas in human genetics. The relationship of cytologically observed nonrandom chromosome changes to neoplasia and genetic diseases is a significant problem. Consistent chromosomal abnormalities, such as the translocation between chromosomes 22 and 9 producing the Philadelphia (Ph^1) chromosome associated with chronic myeloid leukaemia [23] and the translocation between 22 and 8 in some cases of Burkitt's lymphoma [18], could be analysed by this approach to detail specific genomic differences between normal and neoplastic cells. The breakpoint on chromosome 22 is in the same region (*q11*) for both of these translocations. Questions concerning the nature of the breakpoint and the reciprocity of the translocations could then be tackled at the molecular level by sorting and cloning the translocated derivative chromosomes from several Ph^1 positive patients, and Burkitt's lymphoma cell lines.

In addition the large numbers of probes for specific chromosomes isolated and characterised by methods outlined here will eventually help to provide the basis for an extensive human linkage map. The availability of a series of restriction site polymorphisms ordered along the chromosomes serving as linkage markers could allow the detection and antenatal diagnosis of genetic diseases. In view of the potential and simplicity of this approach, the rapidly improving methods for sorting chromosomes by flow cytometry should ensure that chromosome-specific libraries will soon be widely available.

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We thank Dr. Leanne Wiedemann for providing human leukaemic RNA samples and phage cloning arms, Anne Sproul, Rory Sillar and Jane Kellow for technical assistance and Norma Morrison for assistance in cell culture. We gratefully acknowledge Dr. Marie-Claude Hors-Cayla, Dr. Dominique Veil and Solange Heurtz for providing X/22 hybrid cells and Dr. Peter Goodfellow and Dr. Chris Bostock for the hybrid cell line Thy B133R. We thank Dr. Kay Davies, Dr. Bob Williamson and Dr. J. C. Kaplan for encouragement and discussions.

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Human T-Cell Leukemia-Lymphoma Virus (HTLV): A Progress Report

R. C. Gallo, M. Popovic, P. Sarin, M. S. Reitz, Jr., V. S. Kalyanaraman, T. Aoki, M. G. Sarngadharan, and F. Wong-Staal

A. Introduction

Since type-C retroviruses are known to be involved in naturally occurring leukemias of many animal species [26], a similar viral etiology has been sought in human leukemias. Some of the animal models provide important insight for consideration of human leukemias. For example, while most virus-induced animal leukemias and lymphomas are associated with abundant virus production in the tumor cells, bovine leukemia virus (BLV), the causative agent of bovine leukemias, was not detected until the leukemic cells were cultured in vitro (see review by Miller and Van Der Maaten [13]). This brings out the importance of long-term culture of the appropriate target cells for virus detection and isolation. In 1976, our laboratory reported the discovery of a factor termed T-cell growth factor (TCGF) [15]. Following interaction with an antigen, different subsets of mature T cells respond by making and releasing TCGF or making a receptor to TCGF. The TCGF binds to the receptor-bearing T cells and induces cell growth. Addition of exogenous TCGF can maintain growth of activated mature T cells for long periods [6, 26]. When TCGF was added to T cells obtained from patients with mature T-cell leukemias and lymphomas, some cells directly responded without prior activation in vitro [18]. Some of those samples released a retrovirus which we call human T-cell leukemia-lymphoma virus (HTLV) ([19, 20]; Popovic et al., in preparation). The morphology of HTLV is typically type C. Figure 1 shows an electron micrograph of some HTLV particles. Like all retroviruses, HTLV contains reverse transcriptase, has a high molecular weight RNA genome, and buds from cell membranes. It is distinct from all other known animal retroviruses [9, 16, 22, 23] and to date is the only unequivocal human retrovirus. (The retrovirus later isolated independently in Japan [14, 31] and called ATLV is, in fact, HTLV.) Furthermore, it is specifically associated with certain forms of human leukemia and lymphoma [4]. Here we wish to describe some of the new isolates of HTLV and report on some recent findings on the nature and distribution of HTLV and its transmission to and biological effects on normal T-lymphocytes in vitro.

B. Identification of New HTLV Isolates in Established Cell Lines Derived from Patients with T-Cell Leukemias/Lymphomas

Cell lines derived from patients with leukemia/lymphomas of mature T cells from geographically different parts of the world have been established in culture in the presence of TCGF as described previously for normal mature human T cells [15, 26] and neoplastic mature T cells [18]. These cell lines were analyzed for HTLV by (a) competition radioimmunoprecipitation assay (RIPA) for the major core protein p24 [9], (b) indirect immune fluorescence assay (IFA) using highly specific monoclonal antibody for another HTLV antigen, p19 [24], (c) reverse transcriptase activity in the culture fluids, and (d) electron microscopy. In addition to the positive cell lines CR [19]

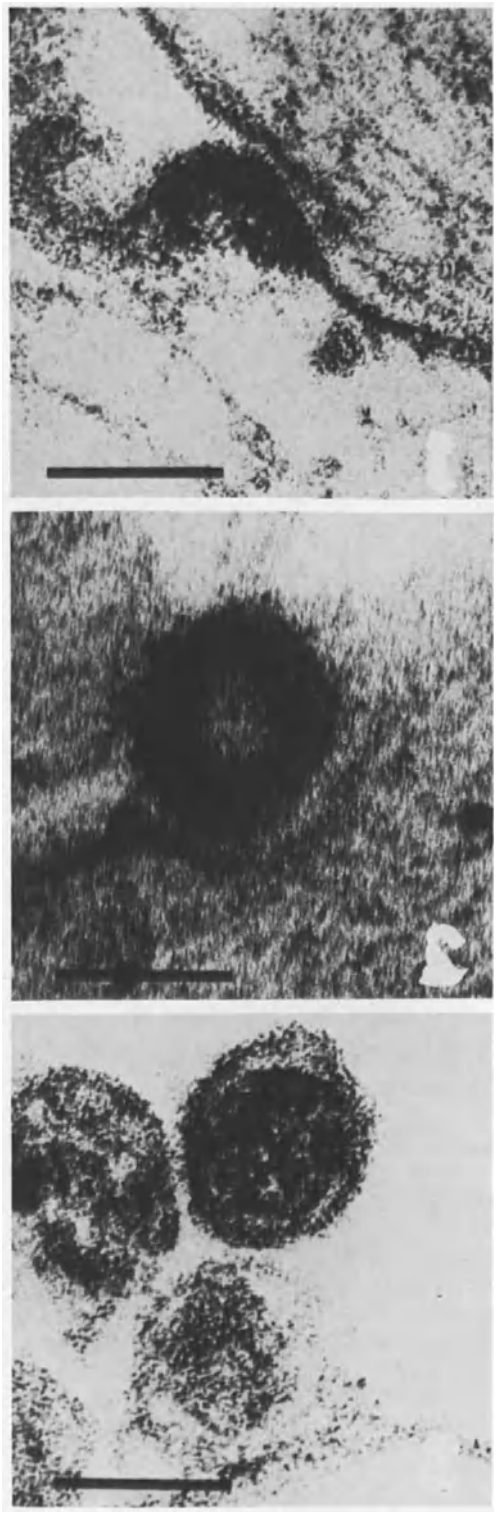

Fig. 1. Electron microscopic examination of the cell line MB, showing extracellular and budding HTLV particles. *Bar* represents 90 μ

and MB [20] published earlier, seven of eight recently established T-cell lines fully expressed HTLV (Table 1) and one showed partial expression. These patients include four individuals from the United States, one from Israel, and three from a single Japanese family from the northwest part of the Honshu Island in Japan. In this family, the patient SK with acute T-cell lymphoma (ATL) and both his parents are virus positive. The father (MK) is clinically healthy and the mother has persistent lymphocytosis, which is considered to be a preleukemic

Table 1. Expression of new HTLV isolates in T-cell lines derived from patients with adult T-cell leukemia/lymphomas

Cell line[d]	p24[a] (ng/mg)	p19[b] (% positive cells)	RT activity[c] (pmol/ml extract)	EM
MJ	128	85	9.3	+
UK	1941	71	4.5	+
MI	1503	63	16.8	+
WA	1076	78	19.2	+
PL	683	23	6.2	+
SK	174	39	5.8	+
TK	2700	54	33.0	+
HK	400	38	13.5	+

[a] Detected by competitive radioimmunoprecipitation assay (RIPA) in cell extract

[b] Indirect immunofluorescence assay (IFA)

[c] Reverse transcriptase activity (RTA) in culture fluids was measured with $(dT)_{15}(rA)_n$

[d] Cell lines were derived from peripheral blood (PB) or bone marrow (BM) of different patients as follows: (a) MJ from PB of a 50-year-old white male with mycosis fungoides, from Boston, Massachusetts; (b) UK from PB of a 45-year-old white male with diffuse histiocytic lymphoma, from Jerusalem, Israel; (c) MI from PB of a 32-year-old black female with T-cell lymphosarcoma cell leukemia, from Granada, West Indies; (d) WA from BM of a 24-year-old black male with diffuse mixed lymphoma, from Augusta, Georgia; (e) PL from PB of a 27-year-old black female with T-cell diffuse mixed lymphoma, from Ovita, Florida; (f) SK from PB of a 21-year-old male with adult T-cell leukemia, from Akita perfecture, Japan; (g) TK from PB of a 45-year-old female (mother of patient SK) who has 7% abnormal cells in her blood, from Akita perfecture, Japan; and (h) HK from PB of a 49-year-old male (father of patient SK) who is normal, from Akita perfecture, Japan

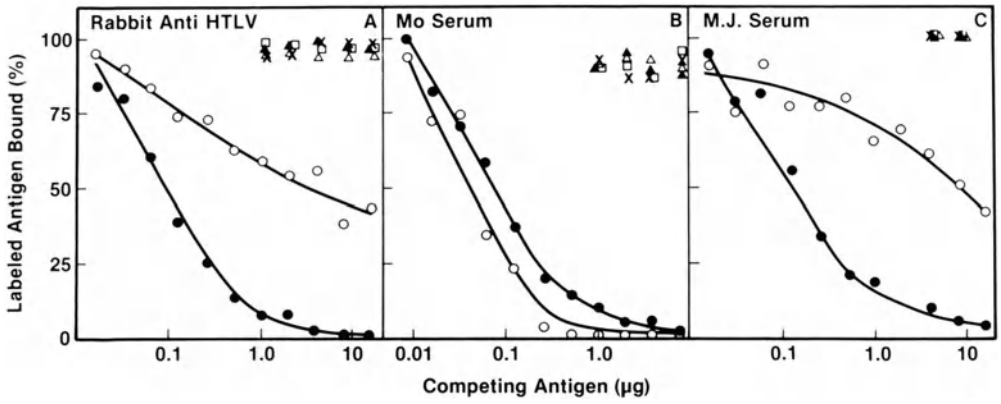

Fig. 2A–C. Homologous and heterologous competition radioimmunoassays of HTLV p24. Assays were carried out as described [9] using ^{125}I-labeled HTLV$_{CR}$ p24 and a limiting dilution of hyperimmune rabbit antibody to HTLV$_{CR}$ or sera from patients MO and MJ. **A** Competition RIA using rabbit anti-HTLV$_{CR}$. **B** Competition RIA using MO serum. **C** Competition RIA using MJ serum. Virus extracts used for competition were: ●–●, HTLV$_{CR}$; ○–○, HTLV$_{MO}$; ×–×, Mason Pfizer monkey virus; △–△, bovine leukemia virus; □–□, Rauscher murine leukemia; ▲–▲, simian sarcoma virus

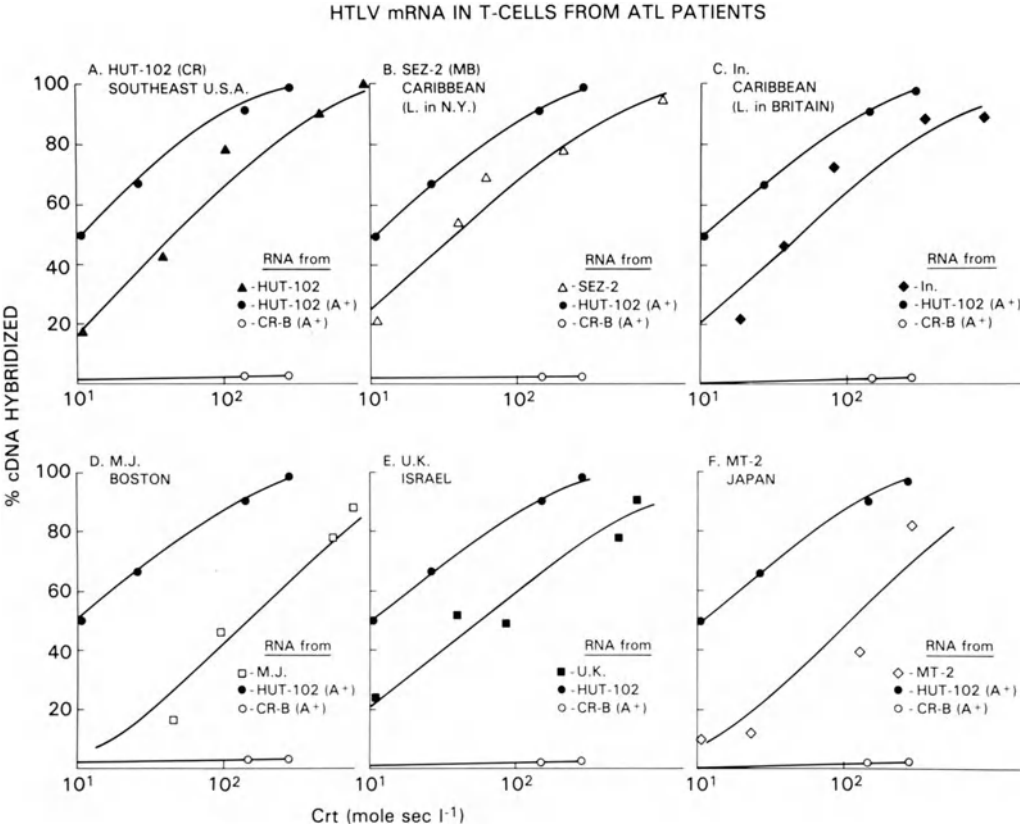

Fig. 3A–F. Relationship of different HTLV isolates by RNA hybridization. ^{3}H-HTLV$_{CR}$ cDNA was synthesized by calf thymus DNA primer [22] and hybridized to cellular RNA from different HTLV-positive cell lines. Results with the controls with poly(A)+RNA from CR-T (HUT 102) and CR-B cells are superimposed on each panel for comparison with the others

state. All cell lines have karyotype and HLA patterns that match those of the donors, and the HLA profiles are different for all eight cell lines. In addition, a T-cell line established by Golde and colleagues from a patient (MO) with hairy cell leukemia [27] was positive vor HTLV p19 and p24 [11]. We propose to designate each virus isolate with a subscript of the patient's initials, e.g., $HTLV_{CR}$, $HTLV_{MB}$, etc. All isolates except $HTLV_{MO}$ are highly related to each other as assayed by competitive radioimmunoassay with p24 (Fig. 2) and hybridization of viral cDNA to mRNA of the producer cell lines (Fig. 3). By these assays, the virus of Japanese ATL is indistinguishable from the prototype HTLV as exemplified by the earlier isolates $HTLV_{CR}$ and $HTLV_{MB}$. On the other hand, $HTLV_{MO}$ competes poorly in the p24 assay (Fig. 2), and nucleic acid sequence homology with $HTLV_{CR}$ was detected only under very nonstringent hybridization conditions (our unpublished data). Therefore, this virus may form a distinct subgroup in the HTLV family. We propose to group them as HTLV-I_{CR}, etc. versus HTLV-II_{MO}.

C. HTLV Provirus in Neoplastic T Cells: Evidence for Exogenous Infection

We had reported earlier that HTLV sequences are present in the infected cells and not in normal uninfected human cells [22], suggesting that HTLV is not an endogenous human virus. In the case of the patient CR, we also had the opportunity to find out whether he was infected pre- or post-zygotically [5]. Several T-cell lines, some clonal derivatives of these lines and a B-cell line have been established from CR. These cells were shown to have originated from the same individual by HLA typing. HTLV proviral DNA was detected in some but not all of the independently established T-cell lines of CR and not in the B cells. An example of the DNA hybridization kinetics is shown in Fig. 4. Furthermore, the surface phenotype OKT3−, OKT4+, and OKT8− appears to correlate with the presence of HTLV. These results indicate that HTLV was acquired by CR by horizontal transmission and suggest that only a subtype of T cells is the target for HTLV infection.

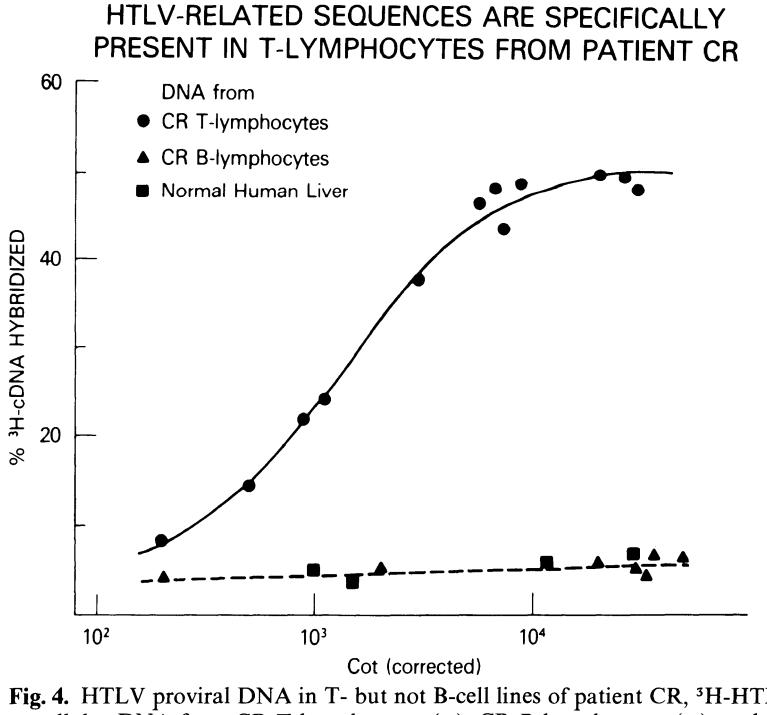

Fig. 4. HTLV proviral DNA in T- but not B-cell lines of patient CR, ^{3}H-HTLV cDNA was annealed to cellular DNA from CR-T lymphocytes (●), CR-B lymphocytes (▲), and PHA-stimulated normal human peripheral blood T-lymphocytes (■)

Recently, molecularly cloned sequences representing the 5′ and 3′ ends of HTLV have been obtained in our laboratory [12a]. These clones have been used as probes for Southern hybridization of fresh leukemic DNA from patients with HTLV-positive diseases [30]. These revealed one or few copies of HTLV integrated at a site which is unique for a given patient but varies from patient to patient. DNA from normal people did not contain hybridizing sequences. A similar observation has been made by others [31]. These results suggest that the infected cells are of clonal origin, so infection must have occurred prior to disease development. This feature is also found in animal leukemia-lymphomas induced by chronic leukemia retroviruses.

D. Clinical Features of HTLV-Positive Diseases

Seroepidemiological studies have identified HTLV-positive patients from many regions of the world with at least three major areas that appear to be endemic: Southwestern Japan [4, 8, 9, 25], the Caribbean [1], central South America (see also Blattner et al., this volume), but only sporadically in the United States [21]. Similar clinical features are found in the diseases associated with these areas, i.e., Japanese adult T-cell leukemia (ATL) and T-cell lymphosarcoma cell leukemia (T-LCL) in the West Indian Blacks from the Caribbean. Both are represented by an aggressive course an frequent association with lymphadenopathy, hypercalcemia, hepatosplenomegaly, and cutaneous manifestations [2, 28]. The tumor cells are all mature, lack terminal deoxynucleotidyl transferase and express differentiated functions. Typing with monoclonal antibodies as well as functional studies showed that the cells may be either of the helper-inducer or suppressor-cytotoxic phenotype. Histologically, the cells are pleomorphic, often with highly convoluted nuclei. Almost all patients with ATL and T-LCL are HTLV positive. These observations led to the hypothesis that HTLV is associated with a subtype of adult T-cell malignancy which may include an aggressive form of cutaneous T-cell lymphoma (CTCL) found in patients CR and MB. In fact, the presence of HTLV may be of practical importance in disease classification. However, at least two HTLV-positive patients have relatively benign diseases: MJ with Sezary syndrome and MO with T-cell hairy cell leukemia. It should be noted, however, that at least the virus in MO is significantly different from the prototype HTLV.

E. Infection and Transformation of Human Cord Blood T Cells by HTLV In Vitro

Seven of the HTLV isolates described above have been successfully transmitted into fresh human cord blood T cells by cocultivation (Popovic et al., in preparation). The virus-positive neoplasic cells used as donors were first treated with mitomycin-C or X-irradiation before cocultivation with recipient cord blood cells. After 4 weeks, assays for T-cell markers, HTLV, karyotype, and HLA-typing were performed. As shown in Table 2, all recipient cord blood are mature T cells, positive for HTLV provirus, and express various levels of HTLV antigens (p19, p24, and RT). Karyotype and HLA typing consistently matched the recipient cells. Since cord blood T cells from the same donors were consistently negative for HTLV markers and the plasma from their cord blood were also negative for HTLV antibodies, we conclude that the virus was transmitted from HTLV-producing neoplastic T-cell lines into cord blood recipient T cells.

To characterize further whether a target for HTLV could represent a certain subset of mature T cells, phenotypes of HTLV-producing cells were analyzed by a series of monoclonal antibodies specific for helper/inducer and suppressor/cytotoxic T cells. We found that a majority of HTLV-producing T-cell lines consistently exhibited only helper-inducer phenotype. Two established T-cell lines, SK and TK, both from Japanese patients and two HTLV-infected cord blood T cells (C1 and C5) revealed "double" phenotype. However,

Table 2. Transmission of HTLV into human cord blood T cells

Cocultured cells (recipient X donor)[a]	Infected cells	Karyotype after coculti-vation[b]	HTLV proteins	Expression of HTLV proteins			EM
				p19 (%) positive cells	p24 (ng/mg)	RTA (pm/ml extract)	
C1(F) X None			–	0	< 1		
C1(F) X MJ(M)	C1/MJ	XX	+	90	385	3.3	+
C4(F) X None			–	0	< 1		
C4(F) X UK(M)	C4/UK	XX	+	81	540	34.1	+
C21(M) X None			–	0	< 1		
C21(M) X MI(F)	C21/MK	XY	+	47	235	60.4	+
C6(F) X None			–	0	< 1		
C6(F) X WA(M)	C6/WA	XX	+	53	502	1.7	–
C8(F) X None			–	0	< 1		
C8(F) X SK(M)	C8/SK	XX	+	47	1000	8.7	+
C7(M) X None			–	0	< 1		
C7(M) X TK(F)	C7/TK	XY	+	75	685	84.2	+
C36(F) X None			–	0	ND		
C36(F) X MO(M)	C36/MO	XX	+	47	500	30.3	ND

F, female; M, male; ND, not done
[a] Mitomycin treated or X-irradiated
[b] 50–100 mitoses were analyzed

none of the T-cell lines exhibited pure suppressor/cytotoxic phenotype. Unlike HTLV-infected cord blood T cells, PHA-stimulated cells (control) consist of 70% helper/inducer and 30% of T cells with suppressor/cytotoxic phenotype. Thus, these data from T-cell phenotype characterization of HTLV-infected T cells again suggest that a certain subset of mature T-cells is the target for HTLV. HTLV infection studies with cord blood cells deprived of T-cell population with helper/inducer of suppressor/cytotoxic phenotype are currently being carried out.

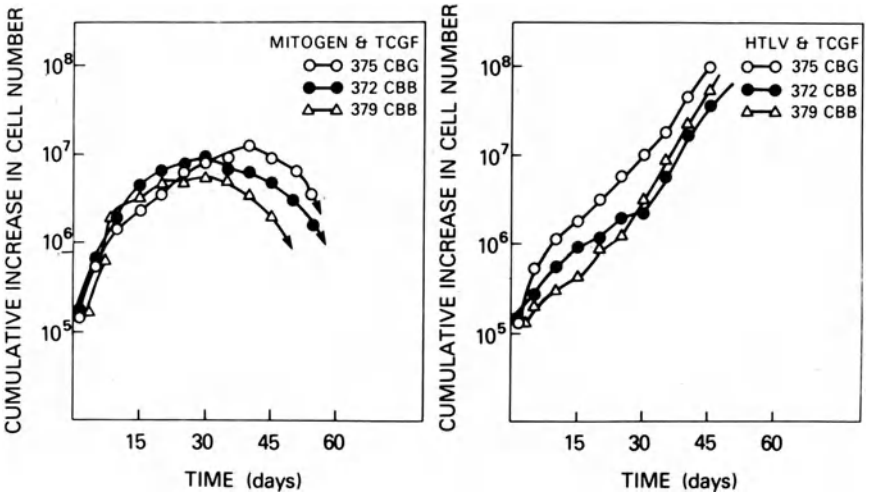

Fig. 5. Growth curves of uninfected and HTLV-infected human cord blood T cells in vitro. *Left panel,* mitogen-stimulated cord blood T cells. *Right panel,* HTLV-infected cord blood T cells. C6/WA and C7/TK cell lines are primary cocultures (for details see Table 2). C5/MJ cells were obtained in three successive transmissions of $HTLV_{MJ}$ isolate into cord blood cells

HTLV-infected cord blood T cells differ from mitogen-stimulated cord blood T cells in several growth properties and cell surface characteristics, the infected cells resembling more the neoplastic cells transformed in vivo by HTLV (see Sarin et al., this volume for details). The most striking feature of HTLV-infected cord blood T cells is their potential for indefinite growth as shown in Fig. 5. In contrast, mitogen-stimulated cord blood T cells from the same patients consistently exhibited growth "crises" after 1 month in culture, even in the continued presence of TCGF. Furthermore, the infected cells, like the neoplastic cells, had the tendency to form clumps in culture. When analyzed by electron microscopy, the cells were seen to have convoluted nuclei (not shown) while the mitogen-stimulated cells did not. Another important and reproducible difference is the decrease in requirement for TCGF by the infected cells. In fact, some of the infected cells are completely independent of exogenous TCGF (see Sarin et al., this volume). Other changes of the infected cells include alteration in their HLA profile and expression of receptors for transferrin, TCGF, and HAA (human-activated lymphocyte antigen detected by monoclonal antibodies) in a high percentage of cells. The data indicate that HTLV is also capable of causing morphological transformation of cord blood T cells in vitro (see Sarin et al., this volume).

F. Possible Molecular Mechanism of Transformation by HTLV

As mentioned earlier, analysis of HTLV-positive leukemic T cells showed that the cells are of clonal origin with respect to the provirus integration sites. In animal systems monoclonality has also been shown to be a common feature of leukemias induced by retroviruses which are chronic leukemia viruses but not those induced by retroviruses which are acute leukemia viruses. Consequently, in spite of its high efficiency to transform T cells in vitro, HTLV probably does not carry on *onc* gene. Several chronic leukemia viruses are known to induce leukemia by activating cellular *onc* genes (*myc* in B-cell lymphomas and *erb* in erythroleukemias) ([7]; Kung, personal communication) by integrating in the proximity of these genes. Activation of these genes is brought about by providing either a viral promotor or viral nucleotide sequences dubbed "enhancer" [12, 17], the real function of which is still unknown. Since HTLV specifically transforms mature T cells, it is likely to affect expression of genes that are important in T-cell proliferation. A model has been proposed for the mechanism of leukemogenesis by HTLV [4]. Briefly, the HTLV envelope protein interacts with the population of T cells normally designed to make TCGF receptors, mimicking an antigen stimulation of blastogenesis. These cells then synthesize receptors for TCGF. Simultaneously, the HTLV provirus integrates in the vicinity of the TCGF gene or a gene that exerts a pleiotropic effect on TCGF expression and activates this gene either by direct promotion or enhancement. The production of TCGF by a cell bearing a TCGF receptor may result in autostimulation and increased cell proliferation. As an approach to study the gene(s) activated by HTLV infection, we have recently identified and isolated a gene that is expressed at high levels in all HTLV-positive neoplastic T cells and in normal cord blood T cells after infection with HTLV but not the uninfected counterparts [12 b]. Study of the expression pattern of this gene in uninfected human hematopoietic cells suggests that its expression may be linked to TCGF production. Experiments are in progress to determine if HTLV integrates at a preferred locus in the human chromosome and affects transcription of specific cellular genes in the vicinity, including this gene in question.

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Early Events in the Suppression of Myeloid Leukemic Cells by Biological Regulators*

D. Metcalf

Culture of mouse myeloid leukemic cells in the presence of a variety of chemical substances can induce the differentiation of some at least of these cells to maturing granulocytes and macrophages. Essentially similar events occur with the human myeloid leukemia line HL60 [1].

Some of these agents, e.g., RNA and DNA inhibitors, are already in clinical use in the treatment of myeloid leukemia, and it may be therefore that part of their action in inducing remissions is not simply by their cytotoxic effects as has been assumed. Karyotypic evidence strongly suggests that, in remission, the repopulating hemopoietic cells are derived from normal precursor cells. However, it is quite possible that during the complex process of remission *induction*, enforced differentiation may play an important role in suppressing the previously dominant leukemic population.

With this increasing awareness of the complexity of action of the process of remission induction, it may be that other chemical agents known to enforce differentiation, e.g., the retinoids, will be tested clinically for their effectiveness as adjuvants in the induction of remission.

A. Normal Regulation of Leukemia Cell Differentiation

Parallel experiments have shown that materials of biological origin are also capable of inducing differentiation in myeloid leukemia cell lines. Without detracting from the potential value of extrinsic chemical substances in controlling leukemia populations, it seems of importance to understand the chemical nature and source of these naturally occurring differentiation-enforcing materials, their function in normal health, and the nature of their action on leukemic cells.

Analysis has shown that in the mouse, three families of specific macromolecules control the proliferation of normal granulocyte-macrophage precursor cells. These are (a) M-CSF of molecular weight 70,000 or 49,000, with a proliferative action essentially restricted to macrophage precursors, (b) GM-CSF of molecular weight 23,000 but of at least two antigenic subtypes with a proliferative action on both granulocytic and macrophage precursor cells, and (c) G-CSF of molecular weight 25,000 with an action primarily but not exclusively on granulocytic precursors.

From the evidence available at present, these same molecules are involved in all experiments that have demonstrated the induction of differentiation in mouse myeloid leukemia cell lines using biological materials. However, the three subtypes of GM-CSF differ sharply in their ability to induce differentiation even using a cell line such as WEHI-3B, which, being a myelomonocytic leukemia, is potentially able to differentiate into either the granulo-

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Dilution of G-CSF	Mean number of normal marrow colonies	Percent differentiated WEHI-3B colonies
1:1	27±1	100± 0
1:2	25±1	100± 0
1:4	26±1	99± 1
1:8	28±1	99± 1
1:16	30±1	98± 3
1:32	25±4	86± 0
1:64	14±2	64±18
1:256	7±1	18±16
1:512	4±1	19± 2
Endotoxin serum 1:6	83±4	100± 0
Saline	0	8± 6

Table 1. Activity of purified G-CSF (DF) in stimulating colony formation by normal marrow cells and differentiation of WEHI-3B leukemic colonies

Assay cultures of 75,000 C57BL marrow cells or 300 WEHI-3B cells contained 0.1 ml of varying dilutions of G-CSF purified from mouse lung conditioned medium. Cultures scored after 7 days of incubation. Control positive cultures contained 0.1 ml of a 1:6 dilution of C57BL post-endotoxin serum

cytic or macrophage pathway. Thus M-CSF appears to be completely inactive in enforcing differentiation; purified GM-CSF does exhibit detectable differentiating activity, primarily into the macrophage pathway; and G-CSF is highly active, forcing cells to enter either the granulocytic or macrophage pathway depending on the experimental conditions used [11, 12]. The high activity of G-CSF in enforcing differentiation has led to the introduction of a second name for this molecule, differentiating factor (DF). It needs to be emphasized, however, that the molecule is a proliferative factor for normal granulocytic precursors and is therefore a G-CSF [15]. Reports [6] that this factor acts only as a differentiation factor and not as a proliferative stimulus are technically erroneous.

Titration of purified G-CSF has shown that the dose-response curves for stimulation of colony formation by normal mar-

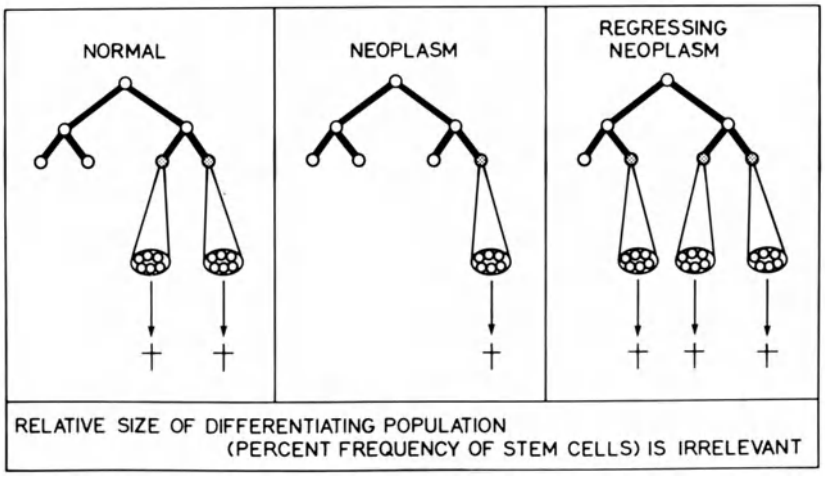

Fig. 1. Schematic diagram indicating that exponential increase in a cancer population occurs when more than 50% of stem cell progeny remain stem cells in nature. Regression of a cancer occurs when this percentage is reduced below 50%

row cells are essentially identical to those for induction of differentiation in leukemia colonies. Thus if the leukemic cells are responsive to differentiation induction, the quantitative aspects of their responsiveness to normal regulators are similar to those of responding normal cells (Table 1).

From the point of view of suppression of a leukemia population the induction of differentiation in even a high proportion of the leukemia cells is of relatively minor importance in determining whether or not the population expands in size progressively. The important question is whether the stem cells in the population continue to generate progeny, more than 50% of which remain stem cells in their proliferative potential. Regression of a leukemia population will only be achieved if the procedure used can force more than 50% of the progeny of stem cells to lose their stem cell properties, either by differentiation or death (Fig. 1).

This question seems not to have been addressed so far in studies of chemical inducers of differentiation, but in the case of biological regulators it has been demonstrated clearly that continuous culture of WEHI-3B cells in the presence of G-CSF (DF) leads to a marked and eventually complete suppression of stem cell self-replication [9]. This appears to be the basis on which DF suppresses the leukemogenicity of myeloid leukemia cells when tested by challenge injection into syngeneic recipients [5, 12].

B. Early Events in Stem Cell Suppression

Because the leukemia cells forming colonies in semisolid agar cultures are demonstrable to be the stem cells in the leukemic population [14], analysis of the ability of leukemia colony-forming cells to self-generate (by recloning individual colony cells) is the most convenient method for monitoring leukemia stem cell self-generation.

Studies of this type have shown stem cell suppression usually to parallel the induction of differentiation in the same clone (colony) [8, 9], but have not determined whether one process precedes the other and whether the *continuous* presence of DF is required for maintenance of stem cells, as is true for the *proliferative* effects of CSFs on normal or leukemia cells.

In recently completed experiments these questions were answered by allowing WEHI-3B leukemia stem cells to divide once or twice in the presence of DF, then, by micromanipulation of washed progeny cells, determining by subsequent culture whether continuing clonogenicity (self-replicative capacity) of the daughter and granddaughter cells could be demonstrated if these cells were cultured subsequently in the absence of DF.

An example of the data obtained for granddaughter cells is shown in Fig. 2. It can be seen that exposure of the stem cells to DF for 18–22 h led to irreversible changes in many of the granddaughters of the stem cells, and in approximately half of the cases one or more of the granddaughters failed to produce a colony on recloning. Analysis of this suppression showed that the affected cell sometimes died without further division but more often divided once or twice in the succeeding 24-h period, after which the cells differentiated prematurely to polymorphs and then disintegrated [13].

These experiments clearly demonstrated that DF is not required continuously to suppress stem cell self-replication but is able to induce an irreversible change that continues to be expressed in the behavior of the progeny of such cells. This process was less evident in the analysis of daughter cells and conversely became more pronounced as clonal expansion increased to the 8–32 cell stage (44 h of incubation), where, for many clones, more than 50% of the stem cells in the clone were suppressed – a formal documentation of the validity of the theoretical proposition shown diagrammatically in Fig. 1 [13]. It will be noted in Fig. 2 that a marked asymmetry was evident in the behavior of the progeny cells formed in the presence of DF. Furthermore, because the cell cycle times of WEHI-3B stem cells are approximately 12 h, the data suggested that one full cell cycle in the presence of DF is sufficient to modify some of the progeny cells permanently. These observations raise the possibility that DF or a product of DF modifies one or more of the daughter chromatids in

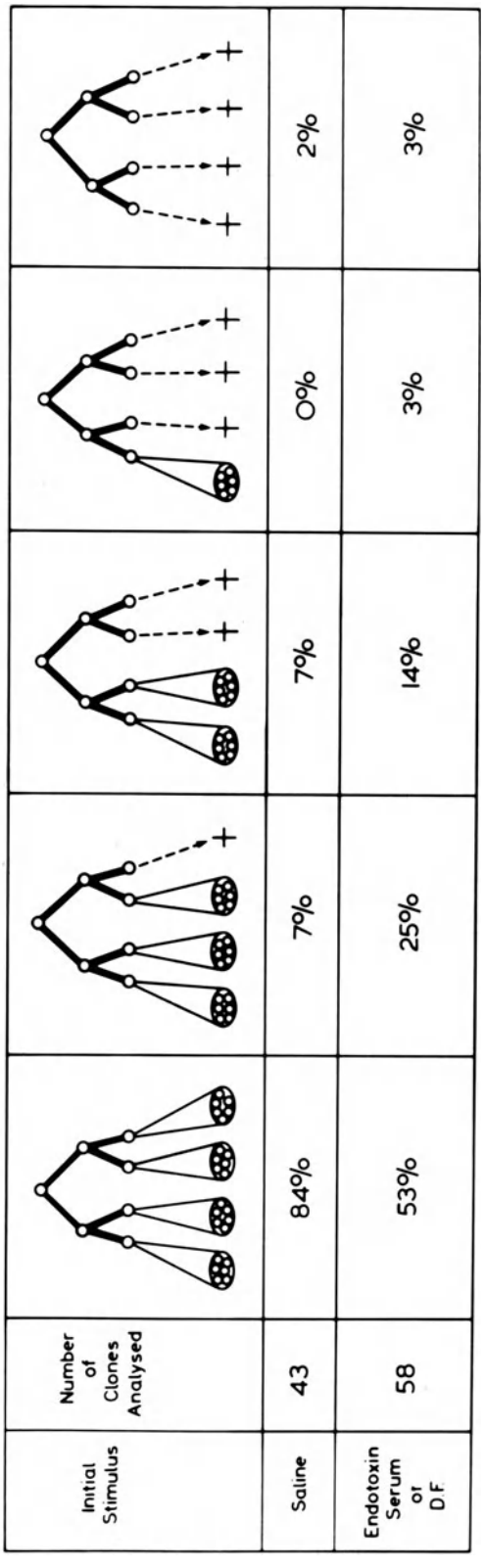

Fig. 2. Effect of presence of G-CSF (DF) during the first two cell divisions of WEHI-3B leukemic cells. Granddaughter cells were washed free of G-CSF (DF) and then individually recloned. Note that after exposure to G-CSF an increased percentage of granddaughter cells fail to generate colonies. In at least two-thirds of these instances suppression is asymmetric

the cell during S phase, possibly by influencing methylation or by insertion of some modulating or promoter sequence during synthesis of the daughter chromatids.

Complete suppression of all stem cells within a treated population appears to be achieved by attrition, with more and more of the progeny stem cells being modified as the exposure period continues.

Since there is clear evidence of heterogeneity in the responsiveness of individual stem cells to the induction of differentiation by DF, this "suppression by attrition" may be based on a similar heterogeneity in susceptibility to suppression. In its extreme form [the so-called differentiation-unresponsive state (D^-)], it has been demonstrated that co-incubation or prior incubation of D^- cells with cytotoxic agents such as actinomycin D renders the cells responsive to the action of DF [4, 16]. Indeed, in at least some cases, the mechanism of induction of differentiation by chemical agents is likely to be indirect, the agents first provoking the leukemic cells themselves to synthesize DF, with subsequent autoinduction of differentiation [3].

C. Sources of G-CSF

The existence of a special type of CSF with exceptional activity on leukemia cells (G-CSF or DF) was established unequivocally by studies on the serum from mice injected with endotoxin [2, 7]. Serum levels of G-CSF rise sharply following the injection of endotoxin and peak 3–6 h after injection [9]. Rises of serum G-CSF have also been observed in mice with acute infections [12].

A search of tissue sources for G-CSF revealed that many organs from normal or endotoxin-injected mice were able to synthesize G-CSF in vitro, in each case the

molecule involved having similar properties and molecular weight [15]. It is still unclear which cell types can synthesize this molecule, but macrophages appear to be one cell with this capacity [13], whereas L cells (fibroblasts) appear to be inactive.

From many published in vitro studies on differentiation induction in cloned mouse myeloid leukemia cells, the impression is created that the critical question is whether the leukemia cells themselves produce, or can be induced to produce, G-CSF. This phenomenon has been documented to occur [3], and at least in the case of WEHI-3B cells the leukemia cells themselves continuously synthesize CSFs of a variety of types. In the context of a leukemia population in vivo, however, it is improbable that G-CSF produced by leukemia cells is a quantitatively important process, and it is more likely that G-CSF production by other tissues is of much more significance.

D. Regulatory Factors Active on Human Myeloid Leukemia Cells

So far relatively little information is available on the existence and activity of naturally occurring regulatory factors for human myeloid leukemia cells. HL60 cells are clonogenic in semisolid agar culture and the colony-forming cells exhibit the capacity of self-replication [17]. It can be assumed therefore that by analogy with the WEHI-3B or M1 systems, the HL60 colony-forming cells are likely to be stem cells in the HL60 population. If this is so, comparable studies on the suppression of HL60 stem cells should be technically feasible using the same in vitro recloning assays used for WEHI-3B cells.

Addition of human placental conditioned medium (HPCM) to cultures of HL60 cells induces morphological changes in HL60 colonies comparable with those seen in differentiating WEHI-3B colonies, although the degree of differentiation induced is less striking than seen following the action of DF on WEHI-3B cells and resembles more closely the weaker action of GM-CSF on WEHI-3B cells (Table 2). This raises the possibility that although HPCM is known to contain two types of human-active GM-CSF (CSFα and CSFβ), neither is analogous with murine G-CSF.

Table 2. Induction of differentiation in HL60 colonies by human placental conditioned medium (HPCM)

Stimulus	Percent differentiated colonies		
	Expt. 1	Expt. 2	Expt. 3
Saline	2	1	2
HPCM 1:16	60	29	50
HPCM 1:32	31	53	19
HPCM 1:64	30	23	17
Human urine	3	9	2
Mouse post-endotoxin Serum 1:6	–	0	0
Mouse GM-CSF	2	4	1

Replicate 2-ml agar cultures contained 1000 HL60 cells and 0.2 ml of the test material. Colonies were scored on day 14

In general, murine CSFs are inactive on normal human GM precursors and murine GM-CSF and DF preparations appear to be unable to induce differentiation in HL60 colonies. Human-active CSFs are active on normal mouse GM precursors, and it is of some interest that HPCM is an effective inducer of differentiation in WEHI-3B colonies (Table 3). Analysis of fractionated HPCM has suggested that CSFα is inactive but that CSFβ (the neutrophilic human active GM-CSF) is active on WEHI-3B cells. The implication from these studies is that at least some human GM-CSFs are active in enforcing differentiation of mouse myeloid leukemia cells. In this context it is of interest that some human sera, particularly from patients with active infections or severe neutropenia, are able to induce differentiation in WEHI-3B colonies [10].

So far as the studies have gone there seems to be little difference in principle between HL60 and mouse myeloid leukemia models, although human analogues of the highly active murine G-CSF have not yet been identified. Since G-CSF is elevated in the serum of mice with active infections, it is possible that elevated serum activity noted in patients with infections may be due to an analogous molecule, although human serum is not particularly

Table 3. Action of human placental conditioned medium in stimulating colony formation by normal mouse bone marrow cells and in inducing differentiation in mouse WEHI-3B leukemic colonies

Stimulus		Mean No. normal mouse bone marrow colonies	Mean percent differentiated WEHI-3B leukemic colonies
Saline		0	4
Mouse post-endotoxin Serum	1:6	100	97
Human placental conditioned medium (HPCM)	1:1	46	63
	1:4	32	40
	1:16	22	26
	1:64	0	19
HPCM CSF$^{\alpha}$	1:1	28	–
	1:4	38	12
	1:16	19	11
	1:64	13	11
HPCM CSF$^{\beta}$	1:1	43	91
	1:4	38	77
	1:16	25	29
	1:64	5	13

Duplicate assay cultures contained 75,000 C57BL marrow cells or 300 WEHI-3B leukemic cells and 0.1 ml of the test material. Colonies scored after 7 days of incubation

promising as a starting material for attempts to characterize biochemically the molecule active on human leukemic cells.

E. Summary

Differentiation of mouse and human myeloid leukemic cells in vitro can be induced by some members of the granulocyte-macrophage family of colony-stimulating factors. In the mouse, the most active molecule (G-CSF) is able to suppress leukemic stem cell self-generation in an irreversible, asymmetric process, suggesting that the factor permanently modifies newly synthesized one or more daughter chromatids in dividing leukemic stem cells.

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Myeloid Leukemic Cell Differentiation Induced by Human Postendotoxin Serum and Vitamin Analogues*

M. A. S. Moore, J. Gabrilove, and A. P. Sheridan

A. Introduction

Fibach and Sachs [11] reported that serum from mice injected with endotoxin induced granulocyte and macrophage differentiation of the mouse myeloid leukemic cell line M1. At that time it was unclear whether the differentiation-inducing factor was CSF (MGI-1). Metcalf [28] also reported that a pure preparation of GM-CSF has some capacity to induce differentiation of murine WEHI-3 myelomonocytic leukemic cells. More recently biochemical characterization of postendotoxin serum has shown that the differentiation factor could be separated from GM-CSF (MGI-1) [24] and was termed MGI-2, or could be separated from the bulk of serum CSF but coeluted with a minor species of CSF that stimulated only granulocyte colony formation [5].

The cellular origin of the differentiation factor (DF) for leukemic cells is diverse (Table 1). Spleen lymphocytes and macrophages have been shown to produce the activity when treated with various mitogens including endotoxin [45]. Conditioned medium from a variety of murine organs also contained DF, but much higher levels of activity were reported following endotoxin treatment [34]. DF produced by the different organs in vitro and found in vivo in endotoxin serum was distinguishable from the majority of granulocyte-macrophage (GM) and macrophage (M) CSF. DF was precipitated by 55% ammonium sulfate and did not bind to Concanavalin A-Sepharose whereas CSF required 85% saturation and bound to Con A-Sepharose. DF also eluted later than CSF from phenyl-Sepharose columns and could be distinguished form M-CSF by its apparent molecular weight (23,000) on native gels [34].

Introduction of DF is radioresistant and independent of T cells since nude mice respond normally [28]. Repeated injections of endotoxin lead to depressed responsiveness as measured by serum DF and CSF levels, and sustained serum DF levels have not been obtained [28]. This is in part due to the short serum half-life of GM-DF (1.5–3 h) and the development of endotoxin "tolerance," which provides a possible major obstacle to the clinical value of endotoxin induction of endogenous mediators.

Endotoxin or other immunostimulants from microorganisms may also induce leukemic cell differentiation in vitro by inducing the leukemic cells to produce endogenous. DF [2, 10, 43]. The structure of micoroorganisms required for a direct effect on the differentiation of leukemic cells and for stimulation of DF production by normal spleen cells may differ since BCG induced direct differentiation of M-1 leukemic cells and spleen DF production, *C. parvum* had no direct effect on M-1 cells but was a potent inducer of spleen cell DF production and *N*-acetylmuramyl dipeptide, and minimal adjuvant-active subunit of the bacterial cell wall had no direct effect on the differentiation of M1 cells and only slightly stimulated the production of D-factor by spleen cells [45].

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Table 1. Sources of GM-DF (MGI-2, D-factor)

Mouse	
Postendotoxin serum	Fibach and Sachs [11], Metcalf [27], Lotem et al. [24], Burgess and Metcalf [5]
Normal mouse organ	Nicola and Metcalf [34]
Lung CM	Maeda and Sachs [25], Metcalf [26], Lotem et al. [24]
Embryo and fibroblast CM	Ichikawa [20], Fibach and Sachs [12], Hayashi et al. [17]
Macrophages	Ichikawa [20], Hozumi et al. [19], Ichikawa et al. [21], Lotem et al. [24], Yamamoto et al. [45]
Stimulated spleen lymphocytes	Hozumi et al. [19], Lotem et al. [24], Akagawa et al. [3], Yamamoto et al. [45]
T-cell hybridoma	Burgess et al. [6]
Ascites from tumor-bearing mice	Hozumi et al. [19], Hayashi et al. [45]
Tumor cell lines	Lipton and Sachs [23], Sachs [41], Yamamoto et al. [45], Moore [30]
Human	
PHA; PWM leukocyte CM	Elias et al. [9], Olsson and Olofsson [35], Olsson et al. [36]
Amniotic fluid	Nagata et al. [33]
Placental CM	Olsson and Olofsson [35]
Postendotoxin serum	Moore et al. [32]

Endotoxin-containing bacterial vaccines have been reported to have therapeutic activity in patients with actue myeloblastic leukemia and nodular lymphoma [22]. In an early study at the Memorial Sloan Kettering Cancer Center conducted from 1970 to 1972, patients with acute myeloblastic leukemia were randomized to receive a vaccine pepared from *Pseudomonas aeruginosa* in addition to chemotherapy protocol. The vaccine was a soluble extract which contained much endotoxin and was intended to prevent or ameliorate *Pseudomonas* infection which pose a serious threat to patients with acute leukemia. While the frequency and severity of *Pseudomonas* infections was not affected by the vaccine, the duration of remission was much longer in the vaccinated group. Six of 13 patients who achieved remission in the vaccine group and 1 of 17 patients in the no-vaccine group are still in complete remission 9 years later. Mixed bacterial vaccine in conjunction with chemotherapy in patients with nodular non-Hodgkins lymphoma also resulted in a higher rate of complete remission, longer duration of remission, and highly significant longer survival [22].

It has become clear that many of the diverse biological effects of endotoxin are not caused by endotoxin itself but by endogenous mediators released by host cells in respone to endotoxin. Three mediators of possible relevance to the antileukemic effects of endotoxin are tumor necrosis factor (TNF), Interleukin-1 (IL-1), and leukemia-differentiating factor (DF); in addition endotoxin is a potent inducer of prostaglandins, interferons, and various species of myeloid colony stimulating factors.

Vitamin A and its analogues (retinoids) also affect proliferation and differentiation of normal and malignant hematopoietic cells. The most extensively studied system has been the human promyelocytic leukemic cell line HL-60 which can be induced to differentiate to mature granulocytes (as measured morphologically, by differentiation antigen expression, and functionally by ability to reduce nitro-blue tetrazolium), following exposure to retinoic acid [4, 18, 36, 38]. Maximum differentiation (approximately 90% of cells) occurs

with 1 μM retinoic acid, a concentration 500- to 160,000-fold less than the concentration of butyrate and dimethyl-sulfoxide that promotes a similar increase in differentiation. Continous exposure to retinoic acid is necessary for maximum differentiation, which occurs after 5 days of incubation, and retinol (vitamin A), retinal acetate, and retinal are approximately 1000-fold less potent than retinoic acid, which can induce some differentiation at concentrations as low as 1 nanomole.

Retinoid induction of myeloid leukemic differentiation is not a universal phenomenon. While the murine myelomonocytic leukemic cell line WEHI-3 can be induced to mature neutrophil differentiation [31] and retinoic acid induces the human malignant monoblast line U937 to monocyte-like cells with the capacity to reduce nitroblue tetrazolium [36], the human myeloid cell lines KG-1 and K562 cannot be induced to differentiate [7]. The mouse myeloid leukemia, M1, can be induced to increase levels of lysomal enzyme production without induction of phagocytosis, locomotive activity, or morphological maturation [43]. Indeed retinoic acid was a potent inhibitor of induction of these latter differentiation-associated properties. Fresh leukemic cells from patients with various myeloid leukemias have also been exposed to retinoic acid in short-term primary suspension cultures and morphological and function maturation was observed only in cases of acute promyelocytic leukemia [14].

The active form of vitamin D_3, 1 alpha, 25-dihydroxyvitamin D_3, and vitamin D analogues may also prove of clinical utility in inducing myeloid leukemic cell differentiation. Abe et al. [1] theorized that since the active form of vitamin D_3 causes multinucleate osteoclasts to appear in bone resorbing surfaces and osteoclasts are thought to be derived from monocytes and macrophages then the murine myeloid leukemia cell line M1 would be a good model to test whether vitamin D can induce this cell line to differentiate into macrophages. The degree of leukemic cell differentiation induced by 12 nM 1 alpha 25-dihydroxyvitamin D was comparable to that induced by 1 nM dexamethasone, the most potent known stimulator of M1 differentiation, and unlike the action of retinoic acid, differentiation-induction included development of phagocytic ability, receptor expression, and locomotive activities. In addition, the vitamin markedly inhibited cell growth in a time-dependent manner. Myelopoietic stimulatition by vitamin D in vitro has been reported by Salahuddin et al. [42], who described a method for the routine long-term growth (greater than 3 months) of normal immature human myeloid cells in liquid suspension culture. The technique employs cell-separated cord blood leukocytes and special growth conditions including hydrocortisone and an obligatory requirement for vitamin D. The augmentation of normal myelopoiesis and inhibition of myeloid leukemic cell proliferation with induction of differentiation seen at comparable in vitro concentrations of vitamin D_3 suggest that in vivo studies may be warranted, particularly since calciferol induces differentiation of leukemic cells refractory to retinoic acid (e. g., M1) and may have significantly fewer side effects at comparable dose levels.

B. Materials and Methods

CFU-c assay: Bone marrow aspirates were obtained from normal volunteers and patients after informed consent. Marrow cells were allowed to sediment, and the leukocyte-rich plasma was collected, centrifuged, and the cells washed twice prior to in vitro culture at 1×10^5 cells/ml in 1.0 ml 0.3% agar in McCoys modified medium containing 10% fetal calf serum. Cultures were stimulated by addition of 10% (v/v) of human GCT cell line conditioned medium (Gibco) as a source of CSF. Cultures were scored at 7 days and the incidence of colonies of more than 40 cells and of clusters of 3–40 cells recorded. Mouse bone marrow cultures of 5×10^4 Balb/c cells were also established in McCoys-agar medium in the presence of WEHI-3 conditioned medium or L-cell conditioned medium as a CSF source.

Leukemic cell lines: Two continuous cell lines of the Balb/c murine myelomonocytic leukemia WEHI-3 were used as murine leukemic targets – the first, reported by Ralph et al. [39] was derived from the 125th

in vivo passage of the B subline of WEHI-3 and is not inducible to differentiate but has the capacity to produce a spectrum of cytokines, particularly many species of CSF [29]. A second cell line was independently developed from WEHI-3B at an early stage of in vivo passage and can be induced to terminal granulocyte and/or macrophage differentiation with loss of self-renewal capacity [26, 30]. To distinguish this line from that of Ralph et al. [39], we have adopted the nomenclature WEHI-3B D^+ for the former and D^- for the latter non-differentiating line. Two cloned cell lines of the human promyelocytic leukemia HL-60 (kindly provided by Dr. R. Gallo, NCI) were used as human leukemic targets. One, termed HL-60 D^-, was originally obtained in 1978 and after prolonged passage lost the capacity to undergo differentiation in response to a variety of inducing agents. The second line, HL-60 D^+, was more recently obtained from a cryopreserved early passage of the original HL-60 and retains neutrophil, eosinophil, and macrophage differentiation potential.

For assay of differentiation induction, titrated serum samples or vitamin analogues were added to 1.0-ml cultures (three plates per point) of 0.3% agar (Difco) in McCoy's modified medium containing 10% fetal calf serum and 300 WEHI-3B, or 1000 HL-60 leukemic cells. Cultures were incubated for 7 days in the case of WEHI-3, or 11–14 days with HL-60 cells, and scored for total number of colonies and total differentiated colonies. Differentiation was characterized by conversion of colonies from compact to diffuse [26]. Diffuse or dispersed colonies reflected differentiation of colony cells to mature neutrophils or macrophages with migratory properties. Additional confirmation of colony differentiation was provided by isolation of individual colonies by pipette, their transfer to glass slides followed by staining with aceto-orcein and microscopic examination.

Suspension cultures of HL-60: HL-60 cells were established in suspension culture at an initial concentration of 5×10^5 cells/ml in 10 ml modified McCoy's medium and 10% fetal calf serum. 13 *cis*-retinoic acid at 10^{-6}–10^{-7} or 1,25-dihydroxycholecalciferol at concentrations from 0.001 μg to 10.0 μg/ml were added at the initiation of the cultures. Following incubation, cultures were assayed at 24 h and 4 and 7 days for total cell count, morphology, and colony-forming capacity upon cloning in agar in the absence of vitamin analogues.

Vitamin analogues: 13-cis retinoic acid and 1,25-dihydroxycholecalciferol were kindly provided by Dr. L. Itri, Hoffmann-La Roche. Analogues were dissolved in absolute ethanol (1 mg/ml) and if not used immediately, were stored at −70 °C in dark vials under nitrogen-saturated conditions.

Human postendotoxin serum: In conjunction with a phase I clinical trial of highly purified endotoxin from *S. abortus* (Novo Pyrexal-Westphal) administered to patients with advanced nonhematopoietic malignancy, we were able to monitor serum levels of CFS and GM-DF. Patients received i.v. injections of endotoxin on a biweekly schedule with escalting doses from 0.1 to 30 mg/m². Bone marrow and serum samples were obtained immediately preceding endotoxin injection and serum at 0.5, 2, 4, 6, 8, and 24 h thereafter. Serum samples were assayed at 10% and 1% v/v against target populations of WEHI-3, HL-60, or autologous marrow cells cloned in agar.

C. Results

Action of vitamin analogues on normal and leukemic cells in agar culture: 13 cis retinoic (RA) and 1,25-dihydroxycholecalciferol (calciferol) were added at concentrations of 10^{-6}–10^{-7} *M* to 1 ml agar cultures containing 1000 HL-60 D^+ leukemic cells, 300 WEHI-3 D^+ leukemic cells, or 10^5 normal human marrow cells stimulated by GCT-conditioned medium. As shown in Table 2, the cloning efficiency of HL-60 cells was 30%, and this was significantly reduced in the presence of either RA or calciferol. Differentiation, as measured by conversion of leukemic colonies from compact to diffuse, was extensive at both molar concentrations of both analogues and approached 100% of colonies at the higher concentration of calciferol. Morphological analysis of individually isolated colonies obtained at 11–14 days revealed that all the control HL-60 D^+ colonies remained undifferentiated blast/promyelo-

Table 2. Comparison of 13-*cis* retinoic acid and 1,25-dihydroxycholecalciferol in the cloning capacity and differentiation of leukemic cell lines and normal human bone marrow

Stimulus	HL-60 D^+		WEHI-3 D^+		Human marrow
	cols/10^3	(% differentiated)	cols/300	(% differentiated)	cols/10^5
Control	307	(0)	60	(9)	179
Calciferol 10^{-7} *M*	143	(45)	39	(55)	363
Calciferol 10^{-6} *M*	63	(91)	16	(83)	442
RA 10^{-7} *M*	237	(77)	56	(48)	194
RA 10^{-6} *M*	186	(87)	62	(93)	227

Table 3. Morphology of HL-60 promyelocytic leukemic colonies exposed to 13-*cis* retinoic acid or 1,25-dihydroxycholecalciferol

Stimulus	Total colonies	% control	% diffuse	Colony Morphology[a]			
				Blast/ prom.	PMN	Macro-phage	G/M mixed
PBS	307	(100)	2	100	0	0	0
10^{-6} *M* retinoic acid	186	(61)	87	0	43	16	41
1 μg calciferol	37	(12)	100	0	40	33	27

[a] Colony morphology determined on 75 individually isolated colonies following orcein staining

cyte in type whereas all diffuse colonies obtained following RA or calciferol exposure were composed of band segmented neutrophils, macrophages, or a combination of both differentiated cell types (Table 3). WEHI-3 D^+ cells had a 20% cloning efficiency, with 9% of these colonies showing some conversion to diffuse morphology in control cultures scored after 7 days (Table 2). RA was only slightly inhibitory to cloning but induced a significant degree of differentiation and conversion of leukemic colonies to diffuse type. Comparable concentrations of calciferol were significantly more effective in inhibiting leukemic colony formation and in inducing WEHI-3 D^+ differentiation (Table 2). In this experiment up to 2.5-fold enhancement of normal human marrow colony formation (exceeding that observed with optimal concentrations of CSF) was seen following addition of calciferol, and a lesser but still significant enhancement was seen with RA. Neither RA nor calciferol was capable of stimulating normal colony formation in the absence of a CSF source. The degree to which RA or calciferol enhanced normal human or mouse colony formation has proved variable from experiment to experiment, and in some cases no augmentation was seen; however, suppression of leukemic colony formation, particularly that produced by calciferol, has been reproducible. Calciferol appeared to be more potent than equivalent molar concentrations of RA in selective inhibition of primary cloning of differentiation-inducible leukemic cell lines. In a further experiment, calciferol (1 μg/ml) was tested for its capacity to inhibit proliferation as measured by colony inhibition, of the nondifferentiating D^- lines of HL-60 and WEHI-3. Only slight inhibition of human and mouse marrow myeloid colony formation (14% and 38% respectively) was seen at this concentration of calciferol (Table 4) whereas the WEHI-3 D^+ line was inhibited 83% and cloning of the WEHI-3 D^- line was still more sensitive, being inhibited 99%. Comparable results were obtained with HL-60 with 99%

Table 4. Comparison of D^+ and D^- leukemic cell sensitivity to colony inhibition by 1,25-dihydroxycholecalciferol

Calciferol 1 μg/ml	WEHI-3 (D^+)		WEHI-3 (D^-)		HL-60 (D^+)		HL-60 (D^-)		Human BM	Mouse BM
	cols/ 300	% diff.	cols/ 300	% diff.	cols/ 10^3	% diff.	cols 10^3	% diff.	cols/10^5	cols/10^5
Control	247	(4)	240	(0)	822	(0)	402	(0)	70	73
Calciferol	41	(73)	3	(0)	11	(100)	0	(0)	60	45

inhibition of cloning of the D^+ line and complete growth inhibition of the D^- (Table 4).

Action of calciferol and RA on suspension cultures of HL-60 cells: In confirmation of numerous earlier studies, the addition of RA at 10^{-7} *M* to suspension cultures of HL-60 D^+ cells initiated at a concentration of 5×10^5 cells/ml resulted in induction of neutrophil differentiation with 26% of cultured cells being differentiated granulocytes by day 6 of incubation in contrast to only 3% in control cultures. No monocyte or macrophage differentiation was seen in RA-stimulated cultures. In contrast, suspension cultures of HL-60 D^+ cells exposed to concentrations of calciferol from 10.0–0.001 μg/ml did not show neutrophil differentiation but did convert to cells of macrophage type. As shown in Fig. 1, calciferol at all concentrations suppressed approximately two to three fold the number of cells recovered after 7 days incubation, but of greater significance was the observation that of the cells recovered by 7 days, 99% were macrophages in the presence of 1 μg/ml calciferol, and even at concentrations of 0.001 μg/ml 78% were macrophages. No neutrophil differentiation was induced. Upon replating 7-day calciferol-exposed HL-60 cells in semisolid agar at 500 cells/ml, in the absence of secondary exposure to calciferol, a major inhibition of recloning capacity was seen (Fig. 1). In

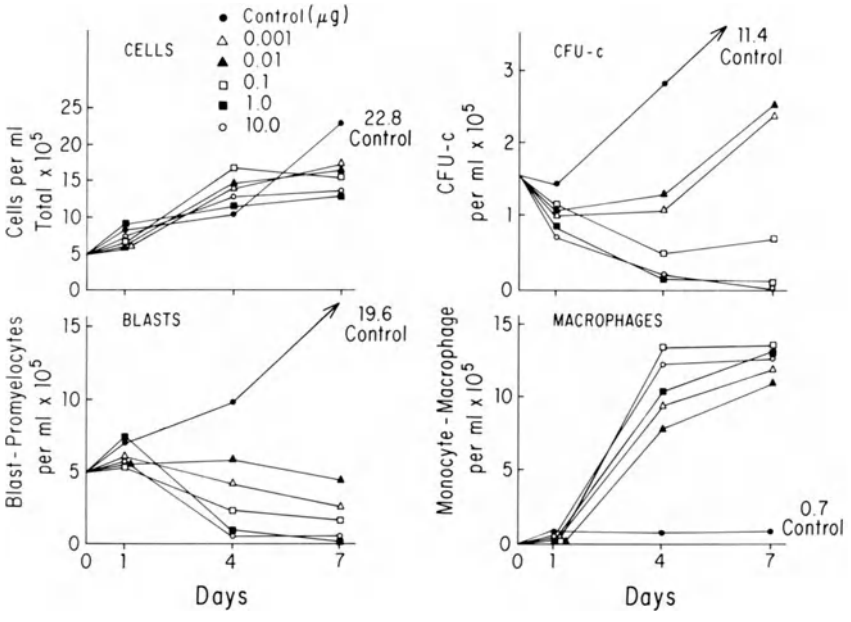

Fig. 1. Ten-milliliter suspension cultures of HL-60 D^+ leukemic cells at a concentration of 5×10^5 cells/ml were exposed to 0.001–10.0 μg 1,25-dihydroxycholecalciferol. At intervals of 1, 4, and 7 days cultures were assayed for cellularity, morphology, and recloning capacity in agar (CFU-c)

contrast to control cultures, which exhibited a 50% recloning capacity, almost complete (99%+) suppression of recloning was seen with HL-60 cells exposed to 1–10 μg/ml of calciferol, and at the lowest concentration of calciferol used (0.001 μg/ml) there was still a 70% inhibition. Colonies that did develop upon recloning of calciferol-treated HL-60 cells were compact and undifferentiated when cultured in the absence of secondary addition of calciferol.

Induction of a leukemia differentiation-inducing protein GM-DF in postendotoxin sera: A highly purified lipopolysaccharide prepared from *Salmonella abortus equi* in a sodium salt form (Novo-Pyrexal) was compared with conventional *E. coli* endotoxin (Difco) for capacity to induce GM-DF in mouse serum following intravenous injection. High titers of GM-DF were induced, peaking between 1.5 and 3 h post-injection with both types of endotoxin. Novo-Pyrexal was somewhat more potent than *E. coli* endotoxin, with detectable GM-DF induction at 0.1 μg. Following a dose of 5 μg/mouse, 3-h postendotoxin sera could be titrated to 1 : 640/1 ml culture of WEHI-3 D^+ cells and still induce leukemic cell differentiation significantly above background, and at a 1 : 20 serum dilution 100% of leukemic colonies were converted to diffuse in gross morphology and differentiated neutrophils and/or macrophages. No inhibition of leukemic colony formation was noted regardless of whether colonies were predominantly diffuse differentiated or compact in morphology.

In conjunction with a phase I clinical trial of Novo-Pyrexal in 25 patients with recurrent or metastatic cancer we were able to assess the capacity of endotoxin in graded doses to induce a human GM-DF in the patients' serum. Novo-Pyrexal was administered by intravenous injection, at doses ranging from 0.002 μg/m² to 55 μg/m². Nineteen patients received multiple doses, and 13 received doses higher than 1 μg/m². No differentiation-inducing activity against either WEHI-3 or HL-60 cells was seen in patient sera collected immediately prior to endotoxin injection, but within 30 min postinjection, a GM-DF activity was detected against both murine and human leukemic cells. The activity peaked between 2 and 6 h and was generally undetectable by 24 h (Fig. 2). At peak activity, up to 90% of WEHI-3 colonies and 50% of HL-60 colonies were induced to differentiate by a 1:10 dilution of the human postendotoxin serum, and GM-DF activity was still detectable at serum dilutions of 1:200. While GM-DF activity in mouse postendotoxin serum was higher than in human, it should be noted that maximum mouse activity was seen following injection of 200 μg endotoxin/kg whereas the human serum activity was obtained following injection of approximately 0.02 μg endotoxin/kg.

The ability of endotoxin to induce CSF in the serum of patients was confirmed by the assay of CSF on the patients' own bone marrow obtained prior to endotoxin injection. As previously reported, normal pre-endotoxin sera failed to stimulate normal myeloid colony formation, but CSF activity was detected 30 min postendotoxin and reached peak levels (272 ± 37 colonies per 10^5 marrow cells) 4–8 h postendotoxin treatment, returning to baseline activity by 12 h.

The reproducibility of endotoxin induction of GM-DF in human serum is illustrated in Fig. 2, which shows the results of injection of 1 μg/m² of endotoxin in five patients with advanced cancer. Induction of WEHI-3 differentiation was particularly marked, with all sera obtained 2–8 h post-endotoxin. An inhibitory activity was also noted in postendotoxin sera, appearing in sera collected within 30 min of endotoxin injection. This inhibitory activity led to reduction in WEHI-3 cloning but did not correlate with the kinetics of induction of GM-DF. An in vivo response to endotoxin was also seen in all patients since the white cell count invariably showed an acute decline within the first 2 h following endotoxin, then proceeded to return to normal levels or to a mild leukocytosis by 24 h (Fig. 2).

Development of pyrogenic tolerance after repeated administration of endotoxin was variable, and in some patients development of tolerance to the leukopenia-leukocytosis effect was also seen. Escalation of endotoxin dosage was effective in overcoming tolerance and biweekly administration of endotoxin to a maximum of 30 μg/m² produced consistent reinduction of serum GM-DF.

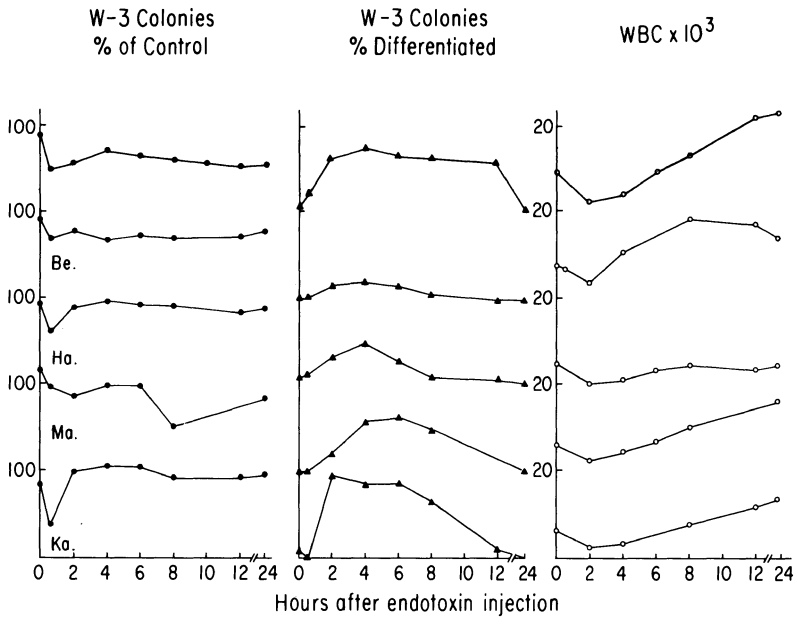

Fig. 2. Induction of a leukemia differentiation inducing factor (GM-DF) in the sera of five patients with advanced cancer following i.v. injection of 1 μg endotoxin (Novo-Pyrexal)/m². Serum DF assayed on a target population of WEHI-3B (D^+) leukemic cells. Note peak induction of differentiation factor 2–4 h postendotoxin. Serum colony inhibitory activity (*left panel*) was maximal 30 min after endotoxin (note, 1–10 μg of endotoxin added directly to cultures of WEHI-3B did not inhibit cloning nor induce differentiation)

D. Discussion

The evidence for a defined endogenous biological entity (GM-DG) with proven capacity to induce, in vitro, the differentiation of a variety of leukemic cell lines and suppress their in vivo leukemia-inducing potential is well established in murine experiments. The present report documents the inducibility of a similar human GM-DF in patients receiving endotoxin. Our ability to reproducibly induce human GM-DF following repeated escalating doses of endotoxin could provide the basis of a differentiation therapy in those leukemias where preliminary in vitro studies suggest a sensitivity to GM-DF. Ultimately, chronic-elevated levels of GM-DF could be maintained by administration of an exogenous source of the factor rather than depending upon injection of an inducer of endogenous activity. There is, however, some evidence to suggest that the multiplicity of endogenous cytokines induced by endotoxin may act synergistically. For example, our studies have shown the endotoxin induces a serum factor which inhibits leukemic cell cloning and differs from GM-DF. Furthermore, the endotoxin protocol induces elevated levels of GM-CSF and Interleukin I, which presumably would be effective in accelerating regeneration of normal myelopoiesis and immune function – particularly following chemotherapy-induced suppression.

The differentiation-inducing ability of retinoic acid has already promoted its use in phase I clinical trials in promyelocytic leukemia and pre-leukemic states [15]; however, antiproliferative action of retinoids upon leukemic cells is both more general than the incidence of induction of terminal differentiation and is seen with retinoid concentrations readily attainable in in vivo. The potential efficiency of retinoic acid in the treatment of human leukemia is further suggested by the observation that

retinoic acid enhances colony-stimulating factor-induced clonal growth of normal human myeloid progenitor cells in vitro [8]. Maximal stimulation occurred at a concentration of $3 \times 10^{-7} M$ retinoic acid, which increased the mean number of colonies by $213 \pm 8\%$ over plates containing CSF alone. Retinoic acid has no direct CSF activity nor does it stimulate CSF production by the cultured bone marrow cells. This stimulation may be mediated by increased responsiveness of the granulocyte-macrophage progenitors and the action of CSF, possibly by increasing the number of CSF receptors per cell.

The ability to sustain chronic levels of retinoic acid in patients with only minor toxicity suggests that this may be a valuable adjunct either to conventional chemotherapy or to additional forms of biological response modification. In this context, leukemia differentiation-inducing factor (GM-DF) produced by mitogen-stimulated human leukocytes acts synergistically with retinoic acid in inducing maturation of the human leukemic lines U-937 and HL-60 [36], and compounds elevating intracellular levels of cAMP, such as dibutryl cAMP, prostaglandin E, and choleratoxin acted synergistically with retinoic acid to induce maturation of both cell lines.

The potential therapeutic value of the active metabolite of vitamin D_3 – 1,25-dihydroxycholecalciferol – in leukemia is supported by our observations on the in vitro response of both D^+ and D^- clones of WEHI-3 and HL-60 cells. In contrast to the action of retinoic acid, calciferol favors a macrophage rather than granulocyte pathway of leukemic cell differentiation, with extinction of leukemic cell self-renewal. In this context, it mimics the macrophage-inducing action of tumor-promoting phorbol diesters [40] and may share the broad spectrum of leukemic cell types responsive to phorbol ester-induced differentiation [37]. In addition to inducing differentiation, low concentrations of calciferol inhibited primary cloning of both D^+ and D^- variants of the leukemic cell lines while even high concentrations were not inhibitory to normal CFU-c; indeed in some studies significant enhancement of normal myelopoiesis was observed. 1,25-dihydroxyvitamin D_3 has proved effective in clinical trials for treatment of patients with postmenopausal osteoporosis [13], suggesting the feasibility of similar trials in patients with leukemia.

A combination of biological response modifiers such as endotoxin-induced GM-DF and vitamin analogues, either alone or as an adjunct to chemotherapy, offers a new approach to treatment of myeloid leukemias and myeloproliferative disorders currently refractory to conventional therapy.

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Effect of Colony-stimulating Factors on the Proteins Synthesized by Normal and Leukemic Myeloid Progenitor Cells*

A. W. Burgess, P. C. Cooper, I. J. Stanley, and N. A. Nicola

A. Introduction

The proliferation and differentiation of normal granulocyte-macrophage (GM) progenitor cells is dependent on the presence of GM colony stimulating factor (GM-CSF). The proliferation of primary leukemic cells is also dependent on GM-CSF, and some established myelomonocytic cell lines (e.g., WEHI3B D^+) can be induced to differentiate by G-CSF.

Using fluorescence-activated cell sorting, it has been possible to isolate highly purified granulocyte-macrophage colony forming cells (GM-CFC) [11]. Fetal liver cells can be labeled with fluorescent pokeweed mitogen and the GM-CFC sorted on the basis of fluorescence intensity and light scatter. We have initiated an analysis of the ^{35}S-labeled proteins in GM-CFC using two-dimensional gel electrophoresis [12]. In particular, the protein changes induced in GM-CFC by macrophage colony stimulating factor (M-CSF) [14], GM-CSF [4], and other hemopoietic regulators [5] have been monitored in an attempt to understand the extent and function of the protein synthetic events associated with commitment to differentiation or proliferation.

Our laboratory has available a murine leukemic cell line [WEHI3B (D^+)] [9] with many similar properties to GM-CFC. A subspecies of CSF (G-CSF), present in the serum of mice treated with endotoxin [3], is able to stimulate WEHI3B (D^+) cells to differentiate to form relatively mature myeloid and monocytic cells. Electrophoretic analysis of extracts from WEHI3B (D^+) cells stimulated by G-CSF has been used to compare the ^{35}S-labeled proteins of these leukemic cells with mature myeloid cells and GM-CFC.

B. Materials and Methods

I. Cells and Culturing

Normal colony-forming cells were purified from murine fetal liver (13–14 day gestation) as described previously [11]. They showed a colony-forming efficiency of 26% and a clone-forming efficiency of 70%–90%.

WEHI3B (D^+) cells were established as a cloned cell line from a mineral oil induced myelomonocytic leukemia (WEHI-3) [15]. Cells were biosynthetically radiolabeled using ^{35}S-methionine (3.7×10^{10} Bq/mmol) (Amersham), at 9.3×10^6 Bq/ml for 3 h at 37 °C in methionine-depleted DME containing FCS (5% v/v) supplemented with methionine (1.3×10^{-5} *M* final concentration). The cells were grown to a density of 10^6/ml for radiolabeling, and after labeling were washed three times with mouse tonicity phosphate buffered saline (MTPBS) before preparation for electrophoresis or nuclear extraction. Bone marrow neutrophils

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were prepared by cell sorting as described previously [16].

II. Preparation of Stimulating Factors

G-CSF was partially purified from ES (50 ml) using gel filtration as described previously [3]. The partially purified preparation of G-CSF was used at 2% v/v final concentration in the experiments to be described. This concentration was a supramaximal stimulus for induction of WEHI3B (D^+) differentiation. Pokeweed mitogen stimulated spleen-conditioned medium [13], GM-CSF from mouse lung conditioned medium [4], and M-CSF from pregnant mouse uterus extract [2] were prepared as described previously.

III. Preparation of Nuclei

Cells were placed into an ice cold hypotonic Tris-HCl buffer (10 m*M* Tris, pH 7.4, 3 m*M* $MgCl_2$) for 5 min (10^7 cells/ml), and lysed using a Dounce glass homogenizer (type B). The disrupted cells were carefully layered over a sucrose solution (0.65 *M* sucrose, 10 m*M* Tris pH 7.4, 3 m*M* $MgCl_2$) and centrifuged at 6000×g for 10 min. Nuclear pellets were resuspended in the hypotonic solution and recentrifuged through 0.65 *M* sucrose.

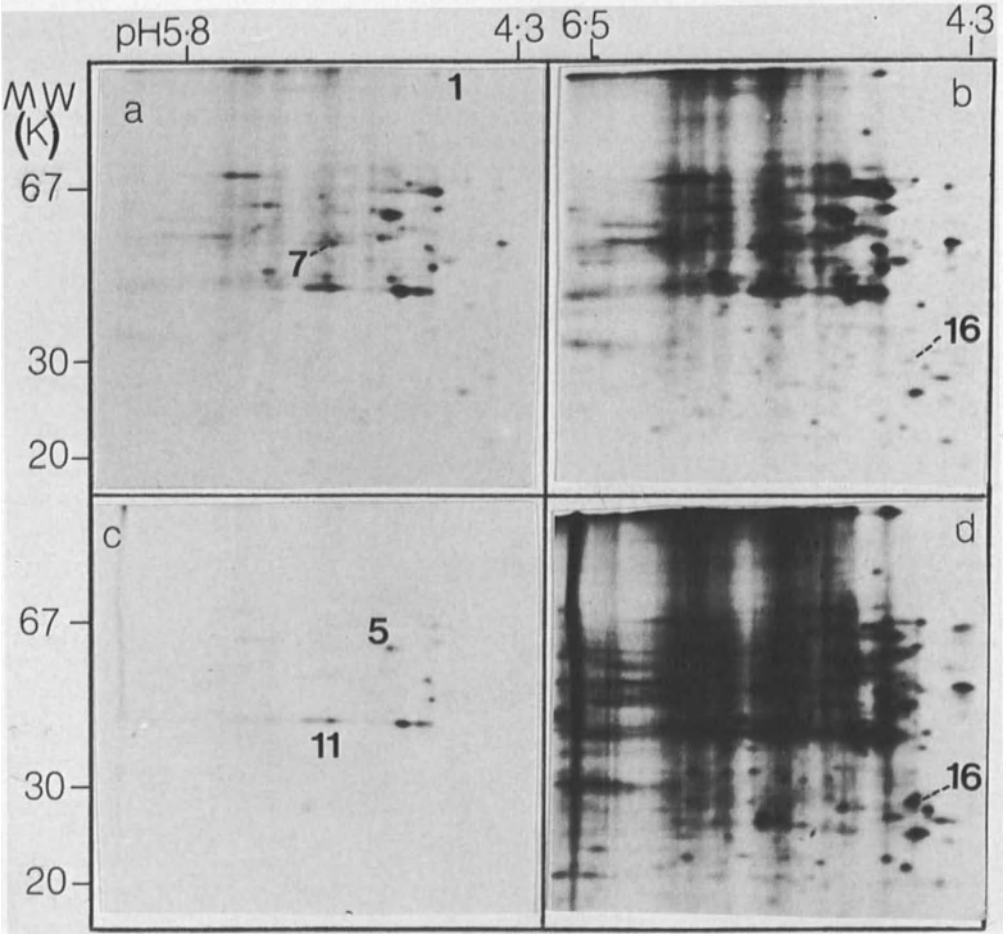

Fig. 1a–d. Effect of spleen-conditioned medium (SCM) on proteins synthesized by purified CFC. **a** Incubation for 18 h with SCM and ^{35}S-methionine (200 μCi/ml); the gel (12%) was processed for fluorography and exposed for 5 h. **b** As for **a** exposed for 20 h. **c** Incubated for 18 h without stimulus in the presence of ^{35}S-methionine (200 μCi/ml) exposed for 20 h. **d** As for **c** exposed for 20 days

IV. Electrophoretic Analysis of ^{35}S-Labeled Proteins

Samples were prepared for two-dimensional electrophoretic analysis by the method of O'Farrell [12]. Radiolabeled proteins from the cells were analyzed using SDS polyacrylamide gel electrophoresis (10% acrylamide cross-linked with 0.25% bisacrylamide) by the Laemmli and Favre [7] procedure. Radiolabeled proteins were detected by fluorography [1].

C. Results and Discussion

I. Protein Synthesis in Colony-Forming Cells

After washing the cells free of the collection medium (PBS-BSA-azide), the enriched colony-forming cells (CFC) were radiolabeled with ^{35}S-methionine for 18 h in the presence or absence of spleen-conditioned medium and the radiolabeled proteins analyzed by two-dimensional polyacrylamide gel electrophoresis (Fig. 1 a–d). The rate of protein synthesis by the CFC in the absence of colony-stimulating factor was considerably reduced (cf., Figs. 1 a, c). In order to compare the relative labeling of particular proteins it was necessary to expose the fluorograms of the proteins from unstimulated CFC for ten times as long as the fluorograms of the proteins from CFC labeled in the presence of CSF. Prolonged exposure of the fluorograms (20 h for the proteins from stimulated CFC and 20 days for the proteins from unstimulated CFC) indicated that most of the proteins in the surviving CFC were synthesized in the same proportion in the presence or absence of CSF (cf., Figs. 1 b, d). However, the small group of proteins near pI 5.5 and molecular weight 23–25K were evident in the unstimulated CFC but not in the proliferating CFC. Similar results were obtained when purified GM-CSF from mouse lung conditioned medium was used to stimulate the CFC.

When the GM-CFC were incubated with another form of CSF, i.e., M-CSF from pregnant mouse uterus extract, the difference between protein synthetic rates of the control and stimulated GM-CFC was evident again (Figs. 2 a, b). Most of the proteins from the CFC stimulated by M-CSF corresponded to those found in both unstimulated and GM-CSF stimulated CFC. However, a chain of proteins with similar molecular weight (near 69K) appeared to be quite sensitive to the type of CSF present. This group of CFC proteins appeared to be undergoing a charge shift (e.g., by phosphorylation) during stimulation and need to be investigated further by labeling the cells with other isotopes,

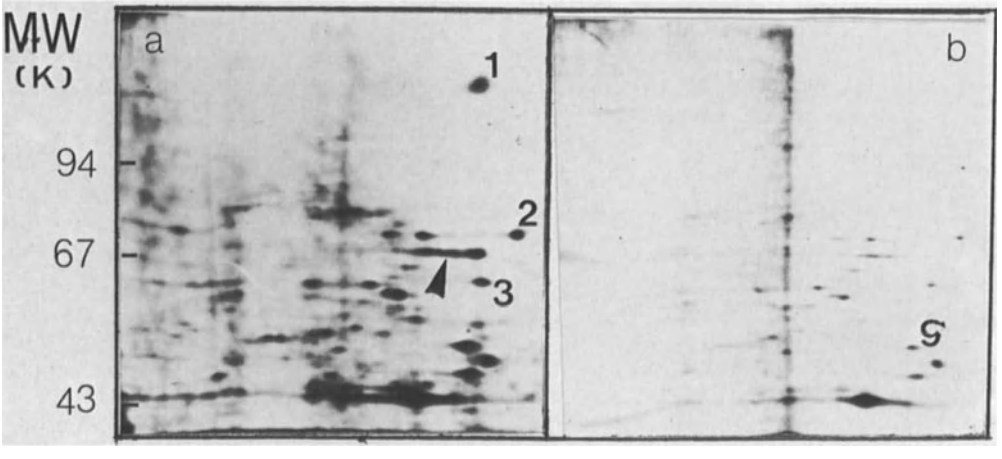

Fig. 2 a, b. Effect of pregnant mouse uterus extract (PMU-E) on proteins synthesized by purified CFC. **a** Incubation for 18 h with PMU-E and ^{35}S-methionine (200 μCi/ml); the gel (8%) was processed for fluorography and exposed for 2 days at – 80 °C. **b** Incubation for 18 h with medium and ^{35}S-methionine and the fluorograph exposed for 5 days

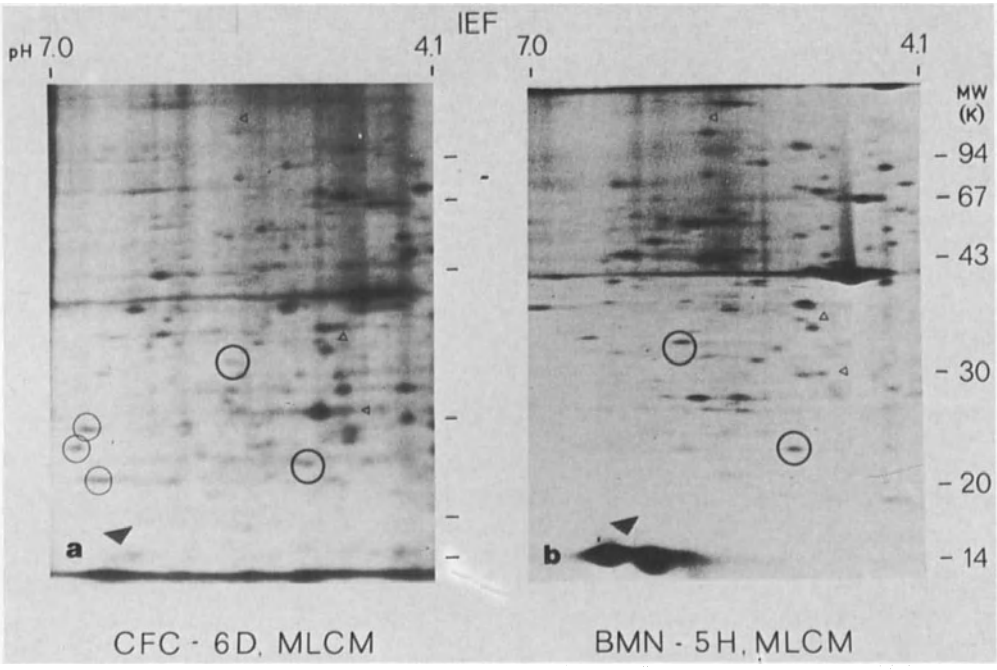

Fig. 3a,b. Comparison of two-dimensional electrophoretic profiles of ^{35}S-labeled proteins from **a** neutrophils produced in vitro by stimulating colony forming cells (CFC) using purified GM-CSF from mouse lung conditioned medium for 6 days and **b** bone marrow derived neutrophils stimulated for 5 h with GM-CSF. Some positions on the profiles are highlighted to allow the proteins to be compared more easily

e.g., $^{32}PO_4$. Again there are remarkably few changes apparent in the relative distribution of protein synthesis in the presence and absence of CSF, especially when previous reports on the protein changes induced by GM-CSF in the M1 leukemic cell line are considered [8].

II. In Vitro and Bone Marrow Derived Neutrophils

When the CFC were allowed to proliferate for 6 days in the presence of GM-CSF from mouse lung conditioned medium, normal myeloid differentiation occurred. The two-dimensional electrophoretic profiles of the ^{35}S-labeled proteins from these in vitro derived neutrophils were compared to a similar profile of ^{35}S-labeled proteins from neutrophils purified from bone marrow (Fig. 3a, b). As expected many of the proteins synthesized by bone marrow neutrophils were also synthesized by the neutrophils derived in vitro. Interestingly the proteins of the bone marrow derived neutrophil induced by GM-CSF were also present in the in vitro derived neutrophils. However, two low molecular weight proteins (approx. 14K) near pH 6 which were rapidly synthesized by bone marrow neutrophils had a very low rate of synthesis in the in vitro derived neutrophils.

III. Myelomonocytic Leukemic Cell Differentiation WEHI3B (D^+)

WEHI3B cells were cultured for 3 h in the presence of G-CSF factors or actinomycin D before being transferred to secondary liquid cultures in the presence or absence of G-CSF or actinomycin D for 24 h. The results of three experiments are summarized in Table 1. Under all culture conditions there was a threefold increase in cell numbers after the initial culture period. When WEHI3B (D^+) cells were cultured initially with G-CSF and then transferred to a culture without G-CSF, myelocytes and promonocytes were still produced (Table 1). Actinomycin D, which has been

Table 1. Initiation of WEHI-3B (D^+) cell differentiation using G-CSF and actinomycin D

Primary stimulus[a]	Secondary stimulus[b]	Cell counts	Percent differentiating cells[c]
Saline	Saline	1.6×10^7	6 ± 3
G-CSF[d]	Saline	1.6×10^7	26 ± 4
G-CSF	G-CSF	1.5×10^7	50 ± 5
Actinomycin D[e] + G-CSF	Saline	1.4×10^7	28 ± 3
Actinomycin D + G-CSF	Actinomycin D + G-CSF	1.3×10^7	68 ± 7
Actinomycin D	Saline	1.5×10^7	29 ± 4

[a] Primary culture was of 3 h duration: there were 4.9×10^6 in 20 ml of culture medium before stimulation

[b] Secondary culture was of 24 h duration

[c] Myelocytes, metamyelocytes, and promonocytes were classed as cells undergoing differentiation. Means ± SD for three experiments

[d] G-CSF was used at 2% v/v final concentration (see materials and methods)

[e] Actinomycin D was used at 5 ng/ml final concentration

shown previously to enhance WEHI3B (D^+) differentiation [6], was no more effective than G-CSF alone in initiating the production of promonocytes or myelocytes (Table 1). The continued presence of either G-CSF or actinomycin D in the secondary culture resulted in a greater degree of WEHI3B (D^+) differentiation. For example, reculture of G-CSF treated cells into medium containing G-CSF resulted in the production of 50% maturing cells, indicating that not all of the cells capable of differentiation in response to G-CSF or actinomycin D were able to be induced within 3 h. Table 1 also shows that actinomycin D enhanced the differentiation when cocultured with G-CSF for the entire culture period (68% differentiation).

Cell cytotoxicity was not found during these experiments, although the actinomycin D marginally decreased the rate of WEHI3B cell proliferation during the culture period. The commitment experiments indicated that events important to cellular differentiation were occurring (in a proportion of cells) within 3 h of treatment. Other data (not shown) have indicated that more cells initiated the differentiation programme after 5 h of treatment with G-CSF than after 3 h of stimulation. Extended culture of the short-term (3 h) G-CSF stimulated cells indicated that many of the cells continued to proliferate, and by 5 days only 10% of the cells were differentiated to any extent. It is still not clear whether the morphologically differentiated cells after 24 h of G-CSF stimulation are irreversibly committed to maturation or whether they can revert to producing blast cells [10].

An examination of the protein changes occurring during the early phases of the differentiation process was performed using two-dimensional gel electrophoresis. WEHI3B (D^+) cells were treated with G-CSF for 2 h and then biosynthetically radiolabeled with ^{35}S-methionine in the presence of G-CSF for a further 3 h. Figure 4 compares fluorographs from untreated WEHI3B (D^+) (Fig. 4a) with the G-CSF treated cells (Fig. 4b). The numbers of the figures indicate the molecular weight of the proteins, which change significantly when the cells are stimulated by G-CSF (letters have been used when two proteins with similar molecular weights need to be distinguished. Six proteins were found to be more heavily radiolabeled after G-CSF treatment (proteins 16a, 18, 35, 66, 85, and a very large molecular weight protein, labeled v1, Fig. 4b). Seven proteins were found to be radiolabeled to a decreased extent (29, 32, 34, 35a, 36, 67, and 84). The decreases in the incorporation of ^{35}S-methionine were found predominantly in acidic proteins (i.e., pI less than 4.8): 29, 32, 34, 35a, 36, 67, and 87. A protein of 16,000 daltons (number 16a in Fig. 4b) exhibited a large increase in label incorporation (6.4 times the amount found in the untreated control cells) (Fig. 4a).

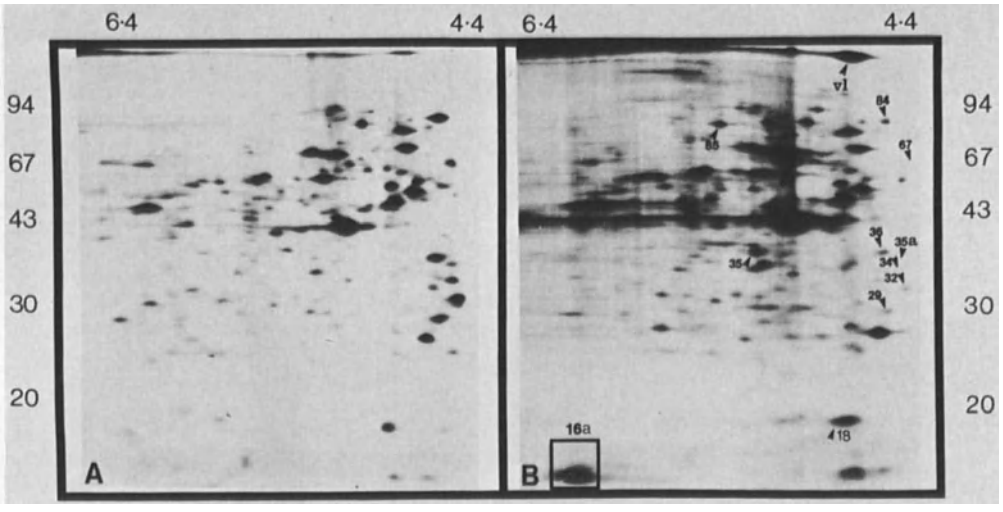

Fig. 4 A, B. WEHI3B cells were treated for 5 h with: **A** NS 2% v/v, **B** G-CSF 2% v/v, radiolabeled during the last 3 h of treatment and then subjected to two-dimensional gel electrophoresis and fluorography. *Arrows* indicate increases or decreases of protein synthesis, induced by G-CSF relative to the NS treated controls. *Numerals* associated with the *arrows* indicate molecular weight of proteins

Examination of the protein changes in the nucleus and cytoplasm of WEHI3B (D^+) at the initiation of differentiation were made using nonequilibrium pH gradient electrophoresis, isoelectric focusing, and SDS gel electrophoresis. The major protein synthetic changes after 5 h stimulation of WEHI3B (D^+) with G-CSF have been tabulated in Fig. 5. The 85K protein was not detected in the nucleus but was present in the whole cell lysates and has been assigned to the cytoplasm. Similarly

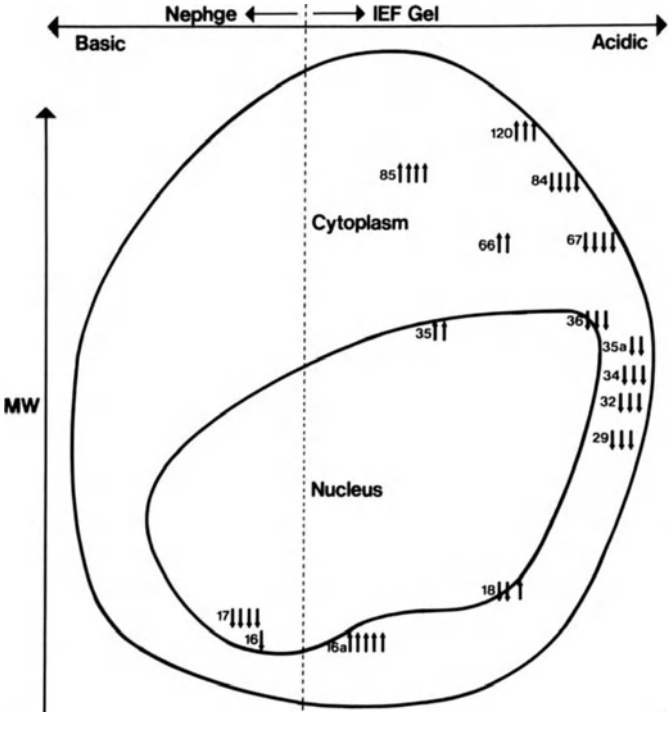

Fig. 5. Summary of changes in protein biosynthesis of WEHI3B (D^+) cells stimulated by G-CSF for 5 h. Subcellular location is indicated by the position of the *arrows* within the compartments. The location of the protein *arrows* on nuclear/cytoplasm boundaries indicates the presence of the protein in both the nuclear and cytoplasmic compartments

the 36K and 35K proteins were assigned to the nucleus because these proteins were more prominent on the nuclear protein profiles than the whole cell lysate profiles. It will still be necessary to examine the cytoplasmic and membrane proteins in more detail before assigning these proteins exclusively to the nuclear compartments.

The 18K protein was detected in the nuclear compartment and decreased 2.6-fold when the cells were stimulated with G-CSF. In the whole cell lysates this 18K protein appeared to increase 1.7–2.1-fold after stimulation by G-CSF. Obviously the analysis may be complicated by both degradation and transfer from one compartment to the other. However, the appropriate pulse chase experiments should be able to clarify the behavior of this protein.

D. Conclusion

Comparison of the protein profiles from the leukemic progenitor cells and normal progenitor cells indicated the presence of specific sets of proteins in common. Interestingly, less than 5 h stimulation of the WEHI3B (D^+) cells with G-CSF induced the production of some of the low molecular weight proteins characteristic of bone marrow neutrophils. Similarly, the synthesis of several of the proteins found in both the GM-CFC and the leukemic blasts was rapidly slowed down when G-CSF was added. Thus, the leukemic cells appear to be capable of an extremely rapid transition from the progenitor to mature cell compartment. Attempts are now being made to understand the function of the proteins common to the GM-CFC and WEHI3B (D^+) which decrease rapidly in response to G-CSF. In particular we are comparing these proteins to known viral oncogene products.

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Molecular Properties of a Factor Inducing Differentiation in Murine Myelomonocytic Leukemic Cells*

N. A. Nicola, M. Matsumoto, D. Metcalf, and G. R. Johnson

A. Introduction

The possibility of therapeutic manipulation of normal regulatory molecules in leukemia has recently gained interest with the demonstration, both in vivo and in vitro, that terminal differentiation and leukemic stem cell suppression can be induced in several mouse myeloid leukemic cell lines by normal tissue products [2–4, 8, 9].

Although a known regulator of normal hemopoietic cell growth, granulocyte-macrophage colony-stimulating factor (GM-CSF) can induce some differentiation in WEHI-3B and M1 leukemic cells [6, 8], another closely related regulator, macrophage-CSF, is devoid of such activity [1, 7]. Moreover, it is becoming clear that the potent differentiation-inducing activity of conditioned media from some cell lines, normal murine tissues, and sera from endotoxin-injected mice cannot be accounted for by their content of GM-CSF [1, 5, 7, 12].

In this report we show that a potent differentiation-inducing activity (DF) in mouse lung-conditioned medium (MLCM) can be completely separated from GM-CSF by chemical fractionation. DF has been highly purified and throughout all fractionation procedures remained associated with a granulocyte-CSF activity.

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B. Results

I. Molecular Properties of the Differentiation Factor

The apparent molecular weight of the differentiation factor (DF) in mouse lung-conditioned medium was determined on native gel filtration, gel filtration in 6*M* guanidine hydrochloride, and in 1*M* acetic acid. All methods gave apparent molecular weights for DF between 21,000 and 26,000. A similar value of 21,000 was obtained for DF by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%–15% linear gradient in acrylamide). DF showed considerable charge heterogeneity when chromatographed on a high-performance packing using the chromato-focusing technique (mono P column, Pharmacia). Although this technique is capable of separating proteins with isoelectric point (IEP) differences of less than 0.2, the DF activity was spread over a pH unit (IEP 4.8–5.8). Rechromatography of selected fractions of DF on the same column established that this was a true charge heterogeneity and not due to buffer interactions. Moreover, chromatography of DF on a high-performance ion-exchange packing (mono Q column, Pharmacia) revealed the same degree of charge heterogeneity.

II. Separation of the Differentiation Factor from the Granulocyte-Macrophage Colony-Stimulating Factor

In addition to charge separation, several other preparative techniques could be used to separate DF from GM-CSF. Although

Step	Ratio of CSF/DF activities
Mouse lung conditioned medium	3.40
DF fraction after salting out	0.87
DF fraction after phenyl-Sepharose	0.43
DF fraction after Biogel P-60	0.38
DF fraction after reverse-phase HPLC	0.40

Table 1. Dissociation of DF and GM-CSF activities during the purification of DF

there was partial separation of DF and GM-CSF using chromatography on concanavalin A-Sepharose (DF failed to bind while GM-CSF showed both binding and nonbinding components), this step proved to be of little use because of the low overall purification of DF. As shown in Table 1, there was a very good separation of DF from GM-CSF using salting out chromatography. When this was followed in sequence by chromatography on phenyl-Sepharose the remaining GM-CSF was completely separated from the DF, with a large number of fractions separating the two activities. Nevertheless, there was a biochemically distinct form of CSF that co-chromatographed with the DF. In all subsequent purification steps and analyses the DF activity remained associated with this CSF activity with no evidence of any separation, and the ratio of the two activities remained unchanged. The CSF activity associated with the DF stimulated only a small number of normal bone marrow colonies (5–20 per 75,000 cells) even at plateau doses (compared to 100–150 for GM-CSF) and, in contrast to GM-CSF, stimulated predominantly granulocytic colonies to develop. It is, therefore, designated granulocyte-CSF (G-CSF).

The current purification scheme involves salting out chromatography and phenyl-Sepharose chromatography as the first two steps because these completely separate GM-CSF from DF and result in a good overall purification (nearly 40-fold) with nearly quantitative yield. This is followed by chromatography on Biogel P-60 in 1*M* acetic acid and then reverse-phase high-performance liquid chromatography (HPLC) using a phenyl-silica based column and an acetonitrile gradient. In this step the DF/G-CSF activity was associated with a small single peak of protein and was over 18,000-fold purified relative to the starting mouse lung conditioned medium. It was half-maximally active in both the in vitro WEHI-3B differentiation and normal bone marrow colony formation assays at 10^{-11} – 10^{-12}*M*, paralleling the specific activity of purified GM-CSF and M-CSF. However, stringent criteria of purity on this purified preparation of DF/G-CSF have not yet been performed.

C. Conclusions

The factor in mouse lung conditioned medium inducing differentiation of WEHI-

	DF/G-CSF	GM-CSF
Ammonium sulfate elution concentration in salting out	1.4 *M*	2.4 – 1.6 *M*
Ammonium sulfate elution concentration on phenyl-Sepharose	0	0.75 – 0.5 *M*
Binding to concanavalin A-Sepharose	–	+/–
Isoelectric point	4.8 – 5.8	4.0 – 5.0
Acetonitrile elution concentration on phenyl-silica HPLC	51%	43%
Molecular weight	21,000 – 26,000	23,000

Table 2. Different molecular properties of differentiation factor and granulocyte-macrophage colony-stimulating factor

3B murine myelomonocytic leukemic cells is different from GM- and M-CSF. This has been clearly demonstrated for M-CSF, since antibodies to M-CSF completely failed to inhibit DF activity [1, 7]. In this report it was shown that GM-CSF can be completely separated biochemically from DF and a summary of their different molecular properties is given in Table 2. Although DF appeared as a single peak of activity through most of the purification steps it did display charge heterogeneity, but the reasons for this are not yet clear. DF was highly purified by the fractionation steps described and, throughout, remained associated with a G-CSF activity. Its role in normal hemopoiesis appears to be to stimulate a small subset of granulocyte colony-forming cells. The availability of purified DF/G-CSF should allow the delineation of the mechanism of action of this molecule, the relationship of the leukemic cells to the normal cell subsets, and exploration of the usefulness of such factors in leukemic cell control.

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A Model Scheme for Human Hematopoietic Cell Differentiation as Determined by Multiple Markers of Leukemia-Lymphomas*

J. Minowada, K. Minato, E. Tatsumi, T. Sugimoto, S. Nakazawa, T. Ohnuma, I. Kubonishi, I. Miyoshi, A. Frankel, and R. C. Gallo

A. Introduction

The analysis of leukocyte differentiation has been greatly enhanced by developments in three areas of methodology: Firstly by the establishment of relatively stable permanent hematopoietic cell lines of a variety of origin, secondly by the development of numerous specific polyclonal and monoclonal heterologous antibodies to various leukocyte antigens, and thirdly by the introduction of many functional assays of both hematopoietic progenitor cells and mature leukocyte subsets aided by various new cell culture and cell separation procedures.

We have been primarily interested in characterizing both permanent leukemia-lymphoma cells and fresh uncultured leukemia-lymphoma cells. At the present, we have a total of 74 proven human leukemia-lymphoma cell lines in the laboratory. These lines include T-cell, B-cell, lymphoid precursor, myelomonocyte, erythroid, and histiocytic lineages [6, 7]. The advantages of using leukemia-lymphoma are twofold: (1) individual leukemia-lymphoma presents an expanded monoclonal population and (2) the marker profile reflects an arrested stage of various point of hematopoietic cell differentiation [4, 5]. Furthermore, all antigens found in the leukemia-lymphoma cells are not tumor specific, but these antigens appear to be the normal gene products. In contrast, the normal hematopoietic cell populations are extremely heterogeneous, with a polyclonal population. For this reason, studies on markers of normal immature cells often lead to equivocal findings.

The present report is limited to some aspects of T-cell leukemia-lymphomas.

B. Materials and Methods

I. Cell Lines and Fresh Leukemia-Lymphomas

A total of 74 factor-independent leukemia-lymphoma lines were maintained in RPMI 1640 medium supplemented with 5%–10% heat-inactivated fetal calf serum. Details of each cell line establishment and characterization have previously been described [5–7]. Mononuclear cells were prepared by the Hypaque-Ficoll gradient centrifugation for the fresh leukemia-lymphoma study.

II. Multiple Marker Analysis

Multiple marker analysis has been developed in our laboratory [6]. The method includes rosette assay, immunofluorescence assay, enzyme assay, cytochemical assay, and cytogenetic assay. In this study, in addition to the polyclonal rabbit antisera to T-cell antigens, Ia-like antigens, common ALL antigens, myelomonocytic antigens, terminal transferase antigens, EB virus antigens, and immunoglobulin chains, a large battery of murine monoclonal hybridoma antibodies were also used for the immunofluorescence test. Cytogenetic analysis was kindly performed by Dr. A. Sandberg and his associates of our Institute, using various banding methods.

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C. Results and Discussion

The present report is limited to some findings associated with 22 T-cell leukemia-lymphoma cell lines and 24 cases of fresh T-cell leukemia-lymphomas.

I. Identification of the Marker Profile for the Earliest T-cell Differentiation

Two of the 22 T-cell leukemia-lymphoma cell lines (P30/Okubo and MOLT-10) and 3 of the 24 fresh T-cell leukemia-lymphomas were found to exhibit a new type of marker profile. The markers included T-cell antigen (T-Ag), common ALL antigen (cALL), terminal transferase (TdT), and Ia-like antigen (Ia). Definitive B-cell marker (immunoglobulins) and myelomonocyte antigens (MAg) were not detected by these leukemic cells, and thus it was concluded that the marker profile is of T cells.

The thymus antigen as defined by the monoclonal antibodies (OKT-6, Leu-6, and NA1/34) was detected in half the T-cell leukemia lines and in two-thirds of the fresh T-cell leukemias of this phenotype, respectively. The findings suggest that the expression of the thymus antigen is within this early stage of T-cell differentiation. Other antigen systems, such as Inducer/Helper (OKT-4, Leu-3A), Suppressor/Cytotoxic (OKT-8, Leu-2A) are also expressed at such an early stage of the T-cell differentiation. As already described [7], the present finding contradicts in part with the model of human T-cell differentiation scheme reported by Reinherz et al. [8].

II. Five Stages of the T-cell Differentiation

It is conceivable that the phenotype of such earliest T-cell differentiation is based on the malignant T-cell leukemias and hence might be an aberrant expression of the antigen profile, not reflecting its normal counterpart. From studies in the past, however, it is still strongly suggested that an extremely small number of normal T cells may express such a phenotype during or before their differentiation in the thymus. This possibility is further strengthened by the fact that the lymphoid precursor with the phenotype of Ia^+, $cALL^+$, and TdT^+ is assumed to be the cells one step earlier than both T-cell and B-cell lineages.

Figure 1 illustrates the five stages of our T-cell differentiation model. Twenty-two

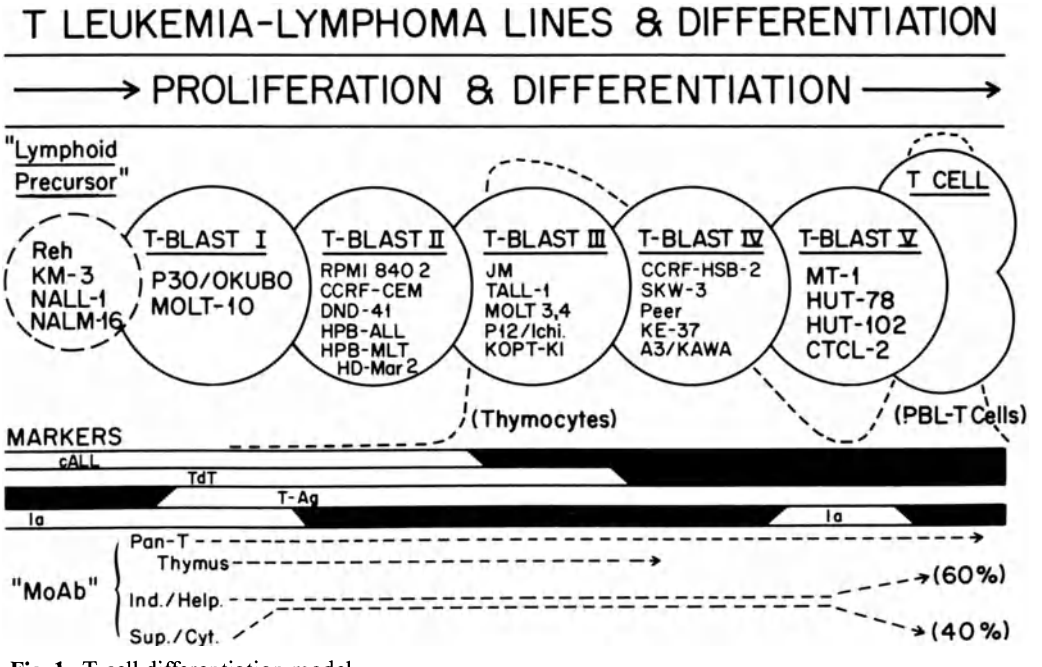

Fig. 1. T-cell differentiation model

T-cell leukemia-lymphoma cell lines are assigned into respective T-blast stages according to individual marker profiles. Four non-T, non-B common ALL cell lines (Reh, KM-3, NALL-1, and NALM-16) are also shown in the "lymphoid precursor" compartment in Fig. 1. Data from analysis with murine monoclonal antibodies (MoAb) are very complicated, and in fact some may not be associated with differentiation as such.

III. Antigens Associated with "Natural Killer Cells" and "Human T-cell Leukemia Virus" (NK- or HTLV-Related Antigens)

Using the monoclonal antibody to an antigen associated with the "natural killer" subpopulation (Leu-7: [1]), three T-cell leukemia cell lines (CCRF-HSB-2, JM, and P12/Ichikawa) among a total of 22 T-cell leukemia-lymphoma lines tested were found to express this "NK" antigen. Interestingly, another monoclonal antibody (AF-45), which had been raised against human prostate cancer cells, reacted only with those three T-cell leukemia lines (CCRF-HSB-2, JM, and P12/Ichikawa). As shown in Fig. 1, while CCRF-HSB-2 is represented in the stage of "T-Blast IV", both JM and P12/Ichikawa are represented in the stage of "T-Blast III". Significance of the finding, however, remains to be determined.

Gallo and his associates [3] have isolated a novel C-type retrovirus from some of the cutaneous T-cell lymphomas. They subsequently developed a monoclonal hybridoma antibody (RG-p19) to a 19,000 mol. wt. viral protein [9]. By the RG-p19 antibody, three T-cell leukemia-lymphoma cell lines (CTCL-2, HUT-102, and MT-1) were found to be positive for the HTLV p19 antigen. All three T-cell lines which had been derived from the Sezary syndrome, mycosis fungoides, and Japanese adult T-cell leukemia, respectively, are represented in the stage of "T-Blast V" (mature T-cells), as shown in Fig. 1. Thus it is of significance that among other possibilities the HTLV infects and manifests itself only in the mature human T cells with the subset phenotype of Inducer/Helper (data not shown).

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Studies on the In Vitro Microenvironment in Man *

J. W. Singer and A. Keating

A. Introduction

The recent adaptation of the murine long-term marrow culture system to humans by Gartner and Kaplan [1] has, for the first time, allowed an examination of the characteristics of those cells that form the in vitro, and possibly the in vivo, microenvironment. Both the human and the murine long-term marrow cultures develop characteristic adherent stromal cell layers that consist of cells described by Dexter as "endothelial-like, fat-containing cells and macrophages" [2]. Until recently, only descriptive information was available about marrow stromal cells (MSC) that are required for the support of in vitro hematopoiesis. We have initiated a series of studies to examine certain properties of MSC and their precursors. These studies suggest that MSC are derived from a transplantable stem cell that is multipotent for other hematopoietic elements. However, MSC also share characteristics with mesenchymal cells previously thought to be unrelated to hematopoietic lineages.

B. Studies in Marrow Transplant Recipients

We examined the origin and some characteristics of MSC in long-term marrow cultures derived from samples obtained from patients who had undergone marrow transplantation following ablative therapy for either leukemia or aplastic anemia. Each of the 14 patients studied received marrow from a sibling of the opposite sex. Marrow samples were obtained between days 14 and 490 after transplantation and used to establish long-term cultures. At 4 weeks after culture initiation, when all cultures had confluent stromal cell layers and were producing CFU-C, supernatant cells were washed off the stromal layer; the adherent cell layer was then treated with trypsin and recultured for analysis of collagen biosynthesis, factor VIII associated antigen, and fluorescent Y body determination [3]. Y body analyses were performed on coded specimens which included normal male and normal female controls. The normal male stromal layers had $66 \pm 18\%$ Y bodies ($n=6$), and the normal female had $1 \pm 1\%$ Y bodies ($n=3$). At least 200 cells were scored for each sample.

The results of this study demonstrated that the marrow stromal cells became increasingly donor derived with time after transplantation. In five of the six informative cases, that is, male patients beyond 40 days after transplantation with female donors, the percentage of Y bodies seen was $\leqq 5$. In three cases, no Y bodies were seen, and in one case, two were seen in 200 cells. Thus, the data indicated that the cells comprising the in vitro microenvironment following marrow transplantation for leukemia become entirely donor derived. In the two patients with aplastic anemia who did not receive total body radiation in this series, one was female and studied at day

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362 posttransplant. Forty-eight percent Y body positive cells were seen in the stromal layer. Since normal male stromal layers had $66 \pm 18\%$ positive cells, 48% is within 1 SD of the male control and thus could indicate that up to 100% of the stromal cells were donor derived.

The observation that MSC were transplantable led us to attempt further characterization. Histochemical stains showed that more than 90% of the explanted stromal cells were peroxidase negative and nonspecific esterase negative. In addition, relatively few cells fluoresced when examined with a monoclonal antibody directed against monocyte-macrophages, the 5F-1. Thus, it appeared that the marrow stromal cells were composed predominantly of cells not in the granulocytic or monocyte-macrophage lineages. An affinity purified antibody directed against factor VIII associated antigen reacted with 5%–25% of stromal cells [4]. Collagen biosynthetic studies were performed by radiolabeling untreated and recultured stromal cells with tritiated proline and tritiated glycine in the presence of beta-aminoproprionitrile and sodium ascorbate followed by polyacrilamide gel electrophoresis after digestion with purified bacterial collagenase and pepsin. The active synthesis of collagen types I, III, and IV was demonstrated [5]. Type IV collagen is synthesized by smooth muscle cells, endothelial cells, and some epithelial cells, but not by fibroblasts. The presence of factor VIII associated antigen and type IV collagen synthesis was demonstrated in stromal cell layers from transplant patients that were close to 100% donor derived. In summary, the studies on MSC following marrow transplantation demonstrated that these cells were transplantable, had certain characteristics associated with endothelial cells such as type IV collagen synthesis and the presence of factor VIII associated antigen, and suggested that there might be a common stem cell for hematopoiesis and its vitro microenvironment.

C. Studies in Chronic Myelogenous Leukemia

The origin and certain features of the cells forming the in vitro microenvironment were studied in a long-term marrow culture derived from a patient with Philadelphia chromosome (Ph′) positive chronic myelogenous leukemia (CML) who was heterozygous for glucose-6-phosphate dehydrogenase (G6PD). Such studies are possible in patients who are heterozygous for the X-linked enzyme G6PD since only one of two X chromosomes is active in each XX somatic cell. Thus, women who are heterozygous for the usual G6PD gene (Gd^B) and a variant such as Gd^A have two cell populations: one synthesizing type A G6PD and the other, B-type enzyme. Skin fibroblasts cultured from Gd^B/Gd^A heterozygotes with CML show both B and A activities, whereas granulocytes, platelets, red cells, monocytes, and some lymphocytes have only a single enzyme type [6]. These data have been interpreted to indicate that CML is a clonal neoplasm arising in a pluripotent stem cell.

The present patient was a 31-year-old nurse who was found to have Ph′-positive CML 6 years prior to study. She had been treated intermittently with busulfan since diagnosis and was in an early accelerated phase when first studied. At that time she had splenomegaly and night sweats. The blood counts included a white blood cell count of $71.1 \times 10^3/mm^3$; platelet count $292 \times 10^3/mm^3$; and hemoglobin 13.4 g/100 ml. The white blood cell differential included 2% blasts and 5% promyelocytes. The marrow showed fewer than 5% blasts in a hypercellular marrow without fibrosis. G6PD studies showed that the red cells, platelets, and granulocytes were type B. Skin, marrow fibroblasts, and T-lymphocytes had equal amounts of A and B activities. Granulocytic colonies and erythroid bursts grown from peripheral blood and marrow were all type B.

A long-term marrow culture was established from the patient and sequentially ended at 3, 6, and 9 weeks for studies of G6PD, collagen biosynthesis, and factor VIII associated antigen. Neither this patient nor six other patients with chronic phase CML produced CFU-C from long-term cultures beyond 4 weeks. This is in striking contrast to cultures from normal individuals which sustain hematopoiesis in excess of 10 weeks in almost all instances. A possible explanation for this phenom-

enon came from an examination of the stromal cell layer. At 3 weeks, the stromal cells had only type B G6PD. However, the stromal cells were actively synthesizing types I, III, and IV collagen, and between 5% and 25% contained factor VIII associated antigen. At 6 weeks, when hematopoiesis had ceased, factor VIII associated antigen positive cells were no longer seen, and some type A G6PD was found in the stromal cell layer. At 9 weeks, equal activities of A and B G6PD were detected, and only types I and III collagen were synthesized. We interpret these data to indicate that at 3 weeks the MSC were synthesizing collagen, were factor VIII positive, and were derived from the leukemic progenitor. This strongly suggests that there is a stem cell common to hematopoiesis and in vitro MSC. The data at 6 and 9 weeks indicated that fibroblasts rapidly became the dominant cell type in the stromal cell layer. In normal stromal cell layers, type IV collagen and factor VIII positive cells have been observed as late as 13 weeks. Perhaps stromal cells derived from the CML clonal progenitor are defective in vitro and thus unable to regulate the outgrowth of fibroblasts. Previous data have demonstrated that marrow fibroblasts in patients with CML do not arise from the neoplastic clone. This in vitro phenomenon may have an in vivo counterpart in the high frequency of development of myelofibrosis in this disorder.

D. Characteristics of Marrow Stromal Cell Precursors

Almost all committed granulocytic and erythroid progenitors, CFU-C, and the BFU-E, express Ia antigen on their cell surface. Between 90% and 100% are lysed when exposed to a monoclonal antibody directed against the Ia determinant (7.2) and complement [7]. In the mouse, it has been shown that most multipotent stem cells (CFU-S) are Ia negative [8]. We examined the ability of an anti-Ia antibody plus complement-treated marrow specimen to form a long-term marrow culture and to generate CFU-C. One such experiment is shown in Fig. 1. In this experiment, the 7.2 antibody plus complement was cytotoxic to 92% of the CFU-C. However, when the treated marrow specimen was used to generate a long-term marrow culture, a morphologically normal MSC layer was formed and CFU-C regenerated. Over the 10-week life of the culture, the cumulative CFU-C yield was equal to 40% of the complement control. However, despite the fact that the complement was not cytotoxic to CFU-C, the complement-treated culture produced significantly fewer CFU-C than did the untreated control. Nevertheless, this experiment demonstrates the ability of an anti-Ia antibody plus complement-treated marrow to regenerate committed progenitors for over 10 weeks and suggests that the stem cells for both MSC and CFU-C

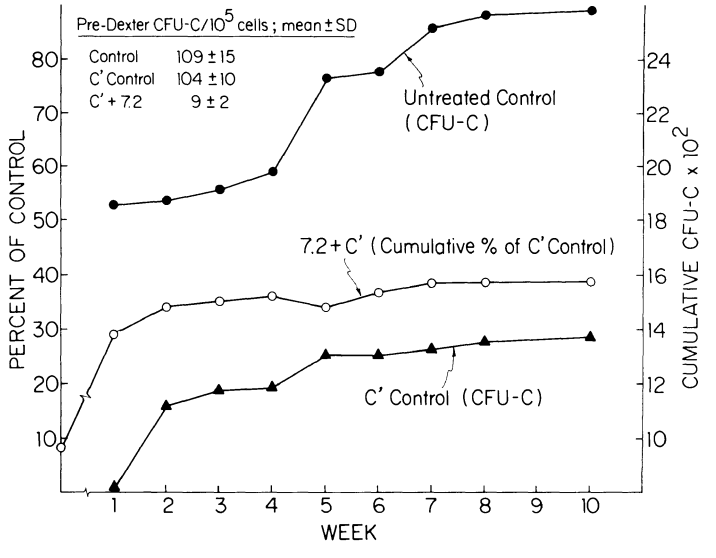

Fig. 1. Normal bone marrow buffy coat cells were treated with a monoclonal antibody directed against Ia-like determinants (7.2) with and without the addition of rabbit complement and then established in long-term cultures. Ninety-two percent of CFU-C were lysed by the antibody with complement; none were lost with the complement control. All cultures generated CFU-C for 10+ weeks. The 7.2 plus complement treated culture generated approximately 40% of the control number of CFU-C over the life of the culture

are Ia negative. Thus, the long-term marrow culture system may be a useful indirect tool for assessing the characteristics of an earlier precursor than those capable of forming colonies in semisolid media.

We found that marrow stromal cells from long-term cultures showed faint fluorescence with two monoclonal antibodies directed against common acute lymphoblastic leukemia antigen (CALLA), the J-5 and the 24.1 ([9], Martin P., personal communication). Studies have shown that both antibodies recognize the same epitope in cross-blocking experiments (Martin P., personal communication). We were also able to demonstrate that the 24.1 plus complement is cytolytic for mature MSC. To determine whether the precursor for MSC also expressed CALLA, we exposed a normal marrow to 24.1 plus complement and then initiated a long-term culture. No differences were noted in the number of CFU-C generated between the complement control and the complement plus antibody-treated specimen. Moreover, normal stromal cell layers developed in both and were subsequently shown to be CALLA positive. This experiment suggested that the precursor cell for marrow stromal cells is CALLA negative and that CALLA arises as a differentiation antigen in MSC.

E. Summary

In a preliminary manner, the data presented here characterize some features of MSC and their progenitors. The progenitors, at least in chronic myelogenous leukemia, are derived from the neoplastic pluripotent stem cell that also differentiates along lymphoid and myeloid pathways. In addition, we have demonstrated that the precursor for MSC is lacking both the Ia and CALLA determinants.

Several antigenic and functional characteristics of the mature stromal cell population have also been identified. Stromal cells express CALLA, synthesize types I, III, and IV collagen, and may express factor VIII associated antigen. It is of interest that fibroblasts do not express factor VIII associated antigen, do not synthesize type IV collagen in measurable quantities, but do express CALLA [9]. Endothelial cells express factor VIII associated antigen, synthesize type IV collagen, but are not CALLA positive. Thus, MSC have some features in common with fibroblasts and others with endothelial cells. The unique characteristics of MSC are that they are transplantable and are derived from a common progenitor with other hematopoietic cells. These features clearly distinguish this cell population from fibroblasts, which are neither transplantable nor derived from the neoplastic clone in CML.

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Granulocyte-Macrophage Progenitor Cell Proliferation and Differentiation

G. E. Francis, J. E. Guimaraes, J. J. Berney, S. Granger, and A. V. Hoffbrand

Cells from both the blood and marrow produce factors which stimulate proliferation and differentiation of granulocyte-macrophage (GM) progenitors in vitro. These two processes are revealed in semi-solid agar cultures by progressively increasing clone size and cellular maturity, and for normal human progenitor cells seem intricately linked. The results of this study suggest that the two processes are, at least in part, individually regulated.

A. Materials and Methods

The culture technique was that of Pike and Robinson [5]. Overlayers contained $0.4–1.0 \times 10^5$ neutrophil-depleted, non-adherent marrow cells (six donors) or $2–3 \times 10^5$ liquid nitrogen stored GM progenitors (from two patients with chronic myeloid leukaemia). Differentiation was assessed from days 3–7 in two ways. First, by morphological and cytochemical analysis of clone cells in dehydrated overlayer gels, using either Wright's stain or a stain for non-specific and chloracetate esterases based on the method of Li et al. [4]. Secondly, since differentiation transitions in the GM progenitor pathway are associated with a progressive loss in proliferative capacity [1], we tested recloning ability, using resuspended cells from two to four pooled day 4 or 7 overlayers, cultured for a further 4–7 days on fresh, blood leucocyte, feeder layers.

Two IgG monoclonal antibodies, OKT3 and OKT11a (Ortho Labs) and one IgM, MBG6 (gift of Dr. A. McMichael) were used with complement lysis to remove populations of T-lymphocytes from marrow or peripheral blood cells [3]. The remaining cells were then incorporated into feeder layers (10^6 cells). Control feeder cells were exposed to antibody or complement alone or were untreated. The $OKT4^+$ and $OKT8^+$ cells used in selective replacement experiments were prepared from blood lymphocytes using affinity columns [6]. The bone marrow endogenous CSA assay and units system are described by Francis et al. [2].

B. Results

Increasing clone cell maturity was followed from day 3–7 of culture by measuring the decline in the proportion of clones containing blasts/undifferentiated cells and the increase in the proportion of clones containing mature neutrophils and macrophages. Depletion of feeder layer T cells using OKT3/MBG6 and complement delayed this process (Fig. 1). Day 3–7 cultures on these feeders showed comparatively immature clones (assessed by morphological/cytochemical characteristics of cells in 200–300 consecutive clones). This difference was obtained irrespective of whether the comparison was made with (a) untreated feeder layers (eight experiments), (b) feeder layers depleted of $OKT11a^+$ cells (11 experiments), or (c) controls: complement alone, antibody alone, complement and irrelevant ascites (ten experiments). Peripheral blood and marrow cell feeders with both fresh or stored target cells showed comparable results and are not shown separately.

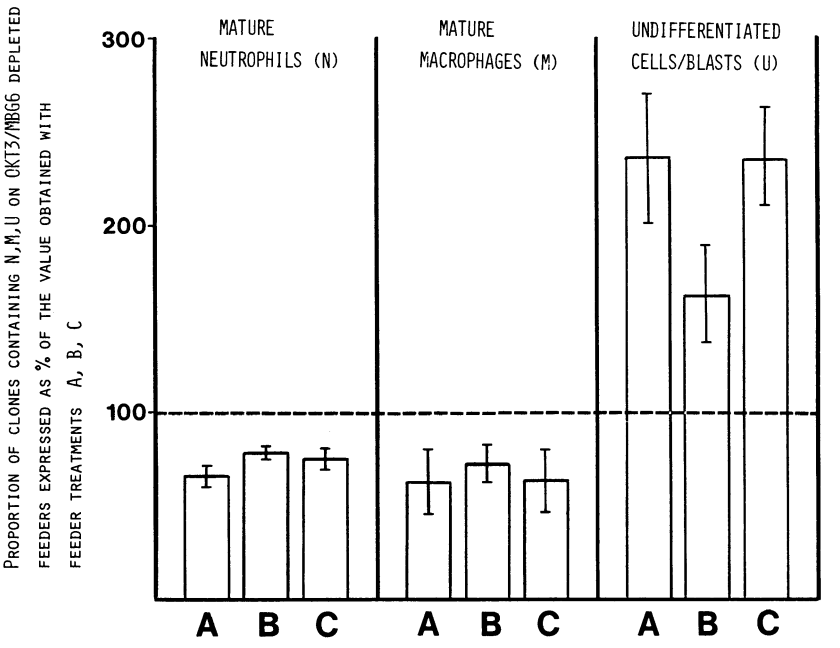

Fig. 1. Depletion of feeder layer T cells, using OKT3/MBG6 and complement, produced relatively immature clone cells compared with feeder layer treatments A, B and C (see text). The differences in the proportions of clones containing N, M and U were statistically significant (Wilcoxon matched pairs signed ranks test, $P < 0.001$)

Serial studies showed that clone numbers were stable from day 3–7 and there was no difference between OKT3/MBG6 depleted, OKT11a depleted and untreated feeder layers (151 ± 5, 151 ± 6 and 157 ± 6 clones/10^4 marrow cells respectively, means $\pm$ SE of five daily values).

Recloning experiments showed that there was an $89\% \pm 28\%$ increase in the number of secondary clones per primary clone when primary cultures were grown on OKT3/MBG6-depleted rather than OKT11a-depleted feeders (the OKT11a-treated controls showed 1ry/2ry clone ratios of 0.65, 0.13 and 0.25, means of three experiments. The secondary clones contained both granulocytes and/or macrophages. Some were large colonies of over 200 cells by day 7 of secondary culture.

Figure 2 shows the effect of replacing the removed OKT3/MBG6$^+$ cells with either OKT4$^+$- or OKT8$^+$-enriched populations. The OKT8$^+$ but not the OKT4$^+$ cells tended to reverse the depression of clone maturity index caused by OKT3/MBG6 depletion. This reversal was only partial; complete reversal would have required an increase of maturity index to 489% of the mean OKT3/MBG6-depleted index value (1.97).

Bone marrow endogenous CSA assays showed that whereas OKT3/MBG6 depletion produced a qualitative change in the stimulus, OKT11a depletion produced a quantitative change. Untreated controls produced 16.0 ± 5.4 units of CSA/10^5 bone marrow cells (mean $\pm$ SE of three samples). OKT11a-depleted marrow produced increased CSA, $231 \pm 37\%$ of control values in three assays. All other treatments produced no significant change in CSA level. At 10^6 cells/feeder all marrows would have provided CSA in excess, stimulating maximal clone formation (> 100 units of CSA).

C. Discussion

The results showed that when feeder layers were depleted of T-lymphocytes bearing the OKT3/MBG6 antigen(s) there was a significant change in the rate of differen-

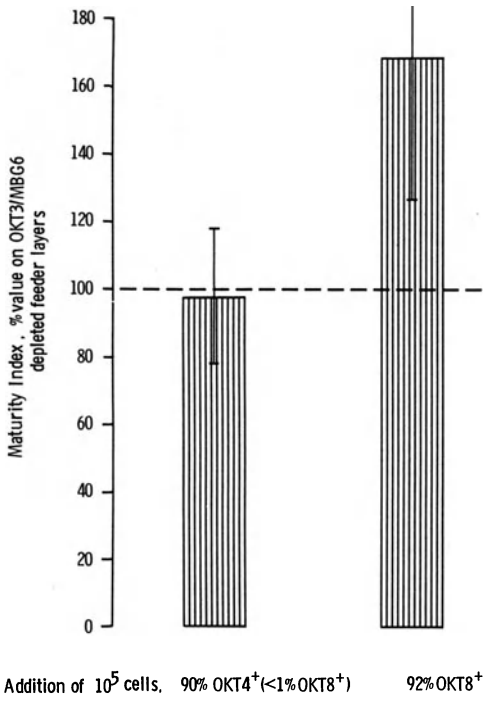

Fig. 2. The effect on clone maturity of replacing removed OKT3/MBG6$^+$ cells with 10^5 OKT4$^+$-enriched or OKT8$^+$-enriched cells. The maturity index = % clones containing mature neutrophils + % clones containing mature macrophages ÷ % clones containing blasts. Means ± SE of three experiments; 100% = 4.4, 1.1 and 0.38 respectively

tiation in the developing GM clones in the overlayer. Replacement of the removed OKT3/MBG6$^+$ cells with OKT8$^+$ cells partially reversed this effect, suggesting that the cells responsible for the selective effect on differentiation have the phenotype OKT8$^+$OKT3$^+$MBG6$^+$. The observation that there was no difference in the number of clones formed on OKT3/MBG6-depleted feeders suggests that the differences observed did not merely reflect recruitment of an additional slowly differentiating clonogenic cell subset, or the failure of clone formation by the most rapidly differentiating cells.

The recloning experiments suggest that the morphological and cytochemical immaturity of the clone cell is accompanied by functional immaturity, with delay in the loss of proliferative capacity which normally accompanies differentiation in the GM progenitor pathway [1].

The contrasting qualitative and quantitative changes in CSA produced by removing OKT3/MBG6$^+$ or OKT11a$^+$ cells presumably result from minority populations bearing only OKT11a (4%–5%) or OKT3/MBG6 (< 1%) antigens.

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Proliferation and Maturation of Hemopoietic Cells from Patients with Preleukemia and Aplastic Anemia in Agar and Diffusion Chamber Cultures

E. Elstner, E. Schulze, R. Ihle, M. Schütt, and H. Stobbe

Aplastic anemia (AA) and hemopoietic dysplasia (HD) (preleukemic syndrome) are diseases which are caused by a defect in the level of hemopoietic stem cells [4, 7, 9, 12] and may result in an acute nonlymphocytic leukemia (ANLL) after a valuable interval. Traditional diagnostic criteria are often insufficient for precise characterization of the above disorders. The present communication aims to illustrate the value of modern proliferation tests (agar colony technique and diffusion chamber technique) for the differential diagnosis and prognostic evaluation of these hemopoietic disorders.

A. Material and Methods

Bone marrow cells of 13 patients with idiopathic acquired AA and eight patients with HD were studied in agar cultures. Diagnostic criteria for HD are presented in Table 1. Bone marrow cells of five patients with idiopathic acquired AA and six patients with HD were examined in diffusion chamber cultures. Bone marrow for controls was obtained from patients without hematological diseases. The cloning of hemopoietic cells was performed in the double-layer agar system described by Pike and Robinson [8]. Colonies (> 40 cells) and clusters (3–40 cells) were scored at day 6–7. For the morphological studies, colonies and clusters were removed from the agar and stained with aceto-orcein.

The other method used for our investigations was the diffusion chamber (DC) technique [1, 3]. The proliferation of human bone marrow cells in DC cultures was estimated on the basis of myelopoiesis occurring in this culture system (proliferation index = I_{prol}).

$$I_{prol} = \frac{\text{number of harvested peroxidase positive cells at day 14}}{\text{number of inoculated peroxidase negative cells at day 0}}$$

(Peroxidase negative cells are known to contain the stem cells). For the morphological investigation smears were stained with May-Grünwald and Giemsa. The maturation of the hemopoietic cells was evaluated by the maturation index (I_{mat}).

$$I_{mat} = \frac{\text{amount of nonproliferative granulopoietic cells at day 14}}{\text{amount of proliferative granulopoietic cells at day 14}}$$

Cells were classified as proliferative granulopoietic cells (blast cells, promyelocytes, and myelocytes) or nonproliferative granulopoietic cells (metamyelocytes, juveniles, bands, and segments).

B. Results

I. Agar Culture

Figure 1 gives the growth pattern of bone marrow from patients with AA and HD in agar culture. The following observations were made:
1. The number of colonies was decreased in both groups of patients (Fig. 1a). But the

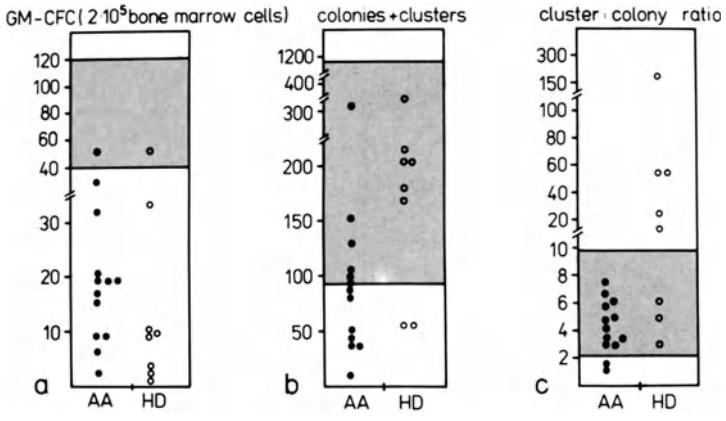

Fig. 1a–c. Growth pattern of bone marrow from patients with AA and HD in agar cultures. **a** Number of GM-CFC. **b** Total count of aggregates (colonies + clusters). **c** Cluster to colony ratio. *Shaded area,* range of 20 normal volunteers

number of total aggregates was decreased only in patients with AA; in HD patients in most cases it was normal (Fig. 1 b).
2. In AA patients the cluster-to-colony ratio was normal. In contrast, in patients with HD the cluster-to-colony ratio was increased in most cases (leukemic type of growth) (Fig. 1 c).
3. The differentiation of the cells in colonies and clusters was normal in patients with AA, but in HD patients with leukemic type of growth the cells of the clusters were very uniform and of a blast cell type.

II. DC Culture

The results of the behavior of the bone marrow cells in DC culture are presented in Figs. 2–4. The following is obvious:
1. The DC proliferation index (I_{prol}) was lowered in patients with AA, but was not decreased in most cases of HD (Fig. 2 a).

Table 1. The preleukemic syndrome: diagnostic criteria

1. Pancytopenia
2. Hyperplastic bone marrow, less than 5% blasts
3. Megaloblastic or/and sideroblastic erythropoiesis with qualitative deviations
4. Either abnormal megakaryocytes or disorderly granulopoiesis (pseudopelger and others)
5. Absence of B_{12} or folate deficiency
6. No treatment with cytotoxic agents in the 3 months preceding diagnosis

2. The DC maturation index (I_{mat}) was not decreased in AA patients; in contrast, it was lowered in all patients with HD (Fig. 2 b).

When comparing the clinical course with the growth pattern in agar and DC cultures we observe the following:
1. The lowest proliferation rates in both assay systems were found for bone marrow from AA patients with a bad course of these diseases.
2. One patient with an extremely high cluster-to-colony ratio in agar culture and low I_{mat} in DC culture developed ANLL 10 months after the investigation.

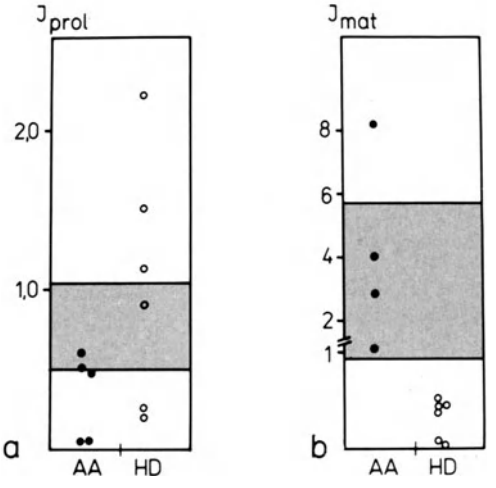

Fig. 2a, b. Growth pattern of bone marrow cells from patients with AA and HD in diffusion chamber cultures. **a** DC-proliferation index (I_{prol}). **b** DC-maturation index (I_{mat}). *Shaded area* range of four normal volunteers

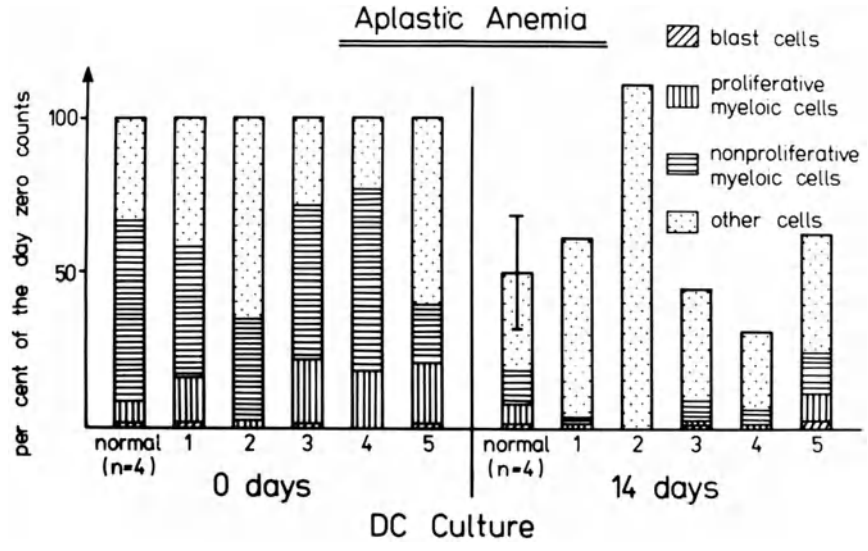

Fig. 3. Differential counts of the day 0 and day 14 diffusion chamber contents

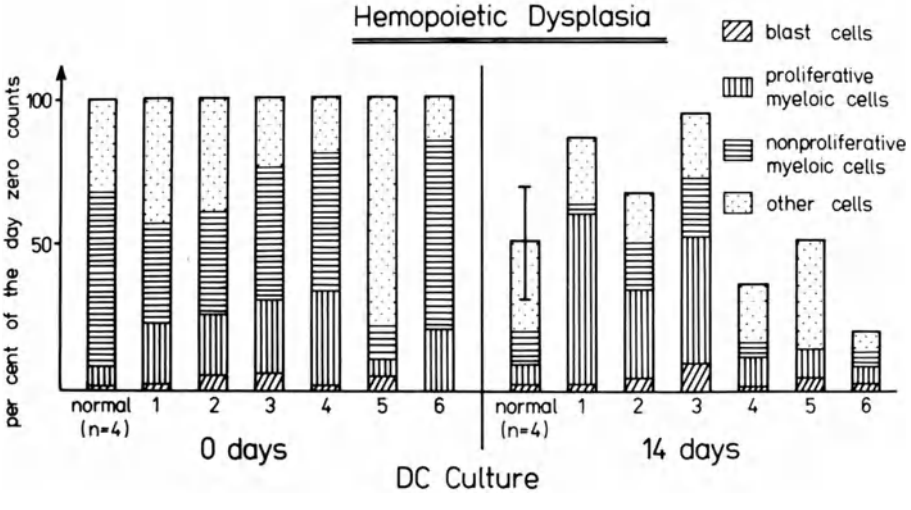

Fig. 4. Differential counts of the day 0 and day 14 diffusion chamber contents

C. Discussion

Precise characterization of AA and HD by means of the traditional diagnostic criteria is often difficult. Our results (Table 2) show that there are distinct differences in the growth behavior of the hemopoietic cells in patients with these disorders. The results of both culture systems point to a true proliferation decrease in the bone marrow of patients with AA: the number of GM-CFC and of total aggregates in agar and the DC-proliferation index were lowered. These findings are consistent with those of other authors, who have noticed a diminished stem cell compartment in AA [2, 5, 6, 10]. The maturation, in contrast, was unimpaired in both systems in this group of patients.

Bone marrow cells from our patients with HD demonstrated an abnormal proliferation in agar culture. This means the

Table 2. Growth pattern in agar and DC culture from patients with AA and HD

	AA	HD
Agar culture		
Number of colonies	↓	↓
Number of total aggregates	↓	Normal
Cluster to colony ratio	Normal	↑
DC culture		
Proliferation index	↓	Normal ↑
Maturation index	Normal	↓

number of colonies was decreased and the cluster-to-colony ratio was markedly increased (Table 2). This in vitro growth pattern is similar to those seen in patients with ANLL. In contrast to the decreased number of colonies the total number of aggregates in agar and the DC-proliferation index were not diminished. Therefore, the reduced number of colonies in the agar culture suggested no proliferation decrease in HD patients, but the establishment of a cell clone with a defective responsibility to CSA in agar [11]. Furthermore, the maturation ability of the hemopoietic cells in HD was strongly affected in both assay systems.

Our results corresponded to the clinical course of the diseases in both systems: the lowest proliferation rates were found for bone marrow of AA patients with a bad course of these diseases; one HD patient with an extremely high cluster-to-colony ratio and a low DC-maturation index developed ANLL 10 months after the investigation.

These observations indicate that growth patterns and maturation ability of bone marrow cells in agar cultures and DC may add to the traditional morphological and clinical criteria for differential diagnosis and prognostic evaluation of these hemopoietic disorders.

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Persistent Deficiency of Myeloperoxidase and Lactoferrin in Granulopoietic Cells of Patients with Acute Leukemia *

K. Rabe, W. Rehpenning, K. Winkler, B. Heinisch, U. Krause, H. Soltau, and R. Neth

Searching for prognostic factors which could help in the management of the therapy of human leukemia, we found marked cytochemical abnormalities in granulopoietic cells in plasma clot cultures of bone marrow and also peripheral blood cells of patients with acute leukemia [1]. We plotted the data of CFU-c colony/cluster ratio [2] versus MPO staining (Fig. 1) and found a significant decrease in the percentage of MPO-positive staining colonies. The colony/cluster ratio also showed a slight decrease, although these differences were not significant. CFU-c colony/cluster ratio versus colony number (Fig. 2) showed no significant difference although a slight decrease in colony numbers was suggestive.

Using these data in a logistic regression analysis and plotting the percentual a posteriori probability of membership to a normal group versus the time after establishing the diagnosis, we found marked abnormalities for leukemia patients on and off therapy (Fig. 4). In order to investigate whether the disturbance of maturation observed in vitro was related to the plasma clot technique or whether it reflected an in vivo mechanism, semiquantitative cytochemical

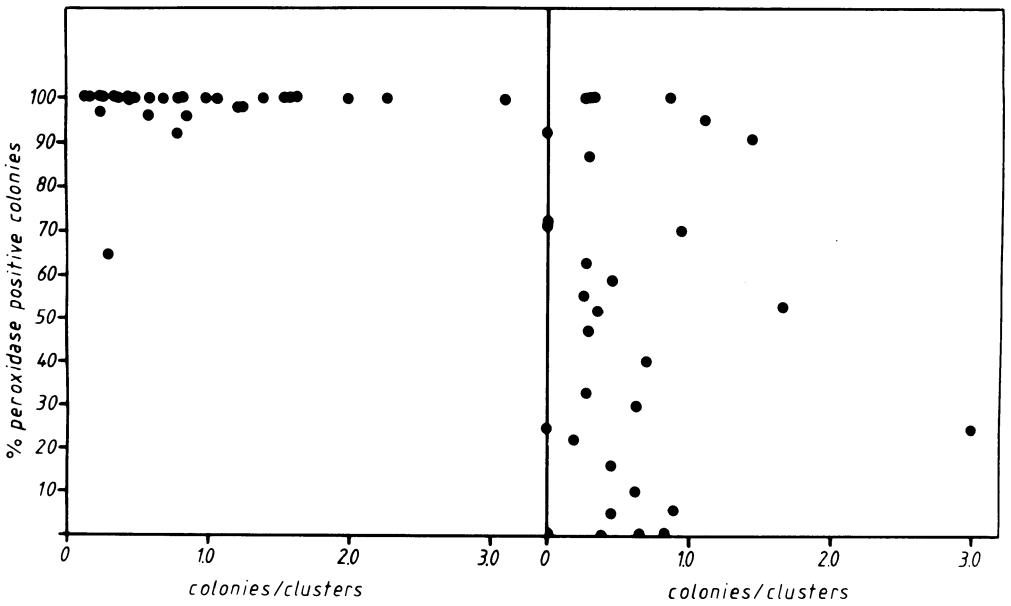

Fig. 1. CFU-c colony/cluster ratio versus MPO staining. *Left,* control; *right,* patient group

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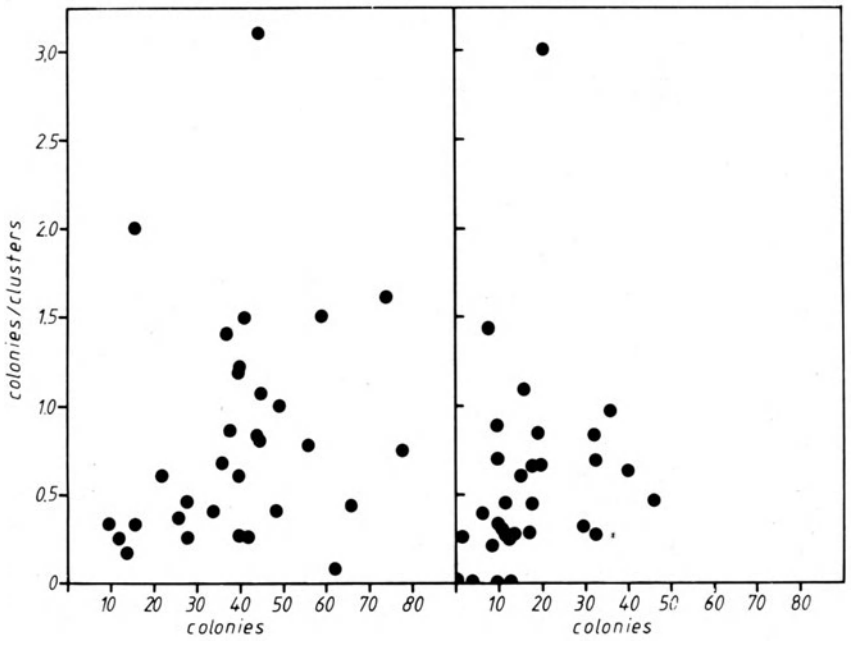

Fig. 2. CFU-c colony/cluster ratio versus colony number. *Left,* control; *right,* patient group

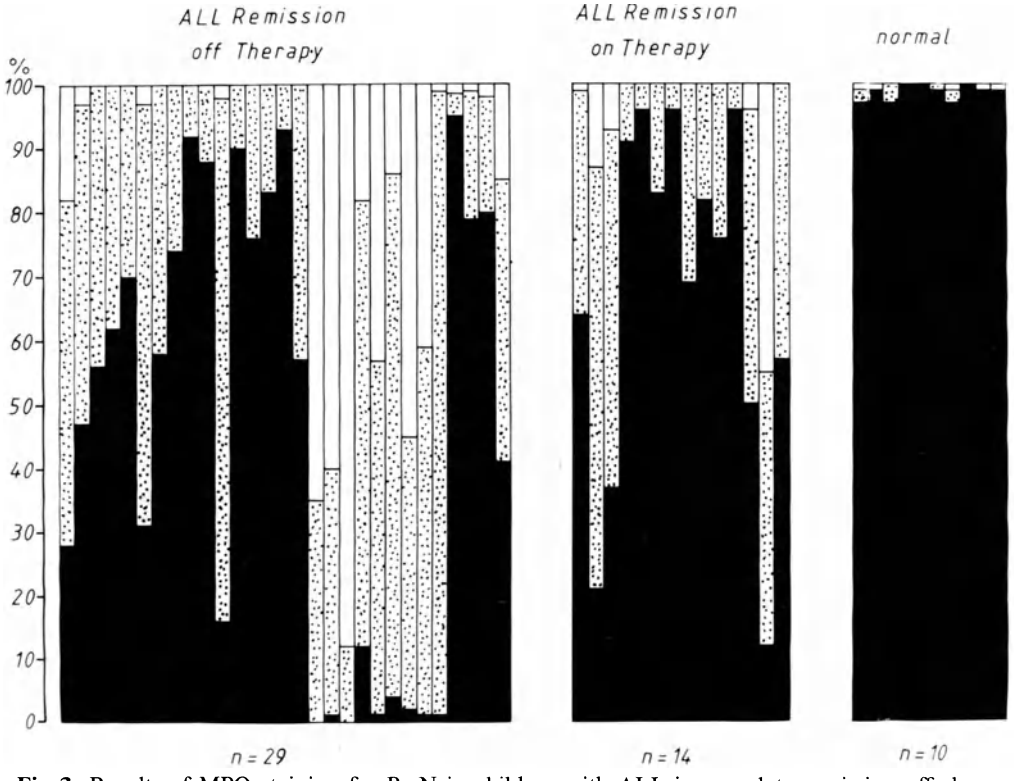

Fig. 3. Results of MPO staining for PmN in children with ALL in complete remission off chemotherapy and on maintenance treatment, and for comparison the juvenile control group. ■, + + cells; ⊡, ± cells; □, – cells

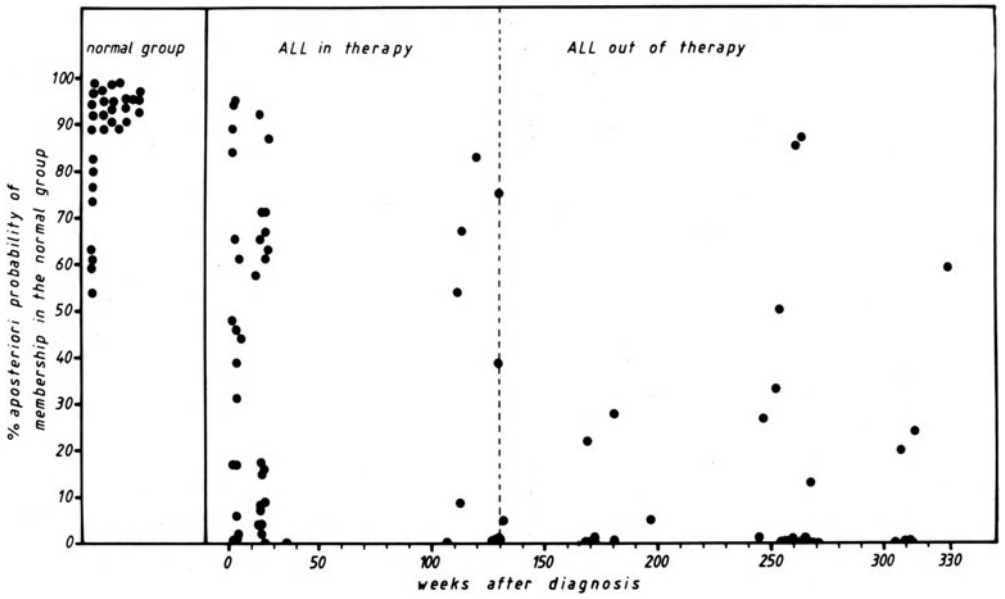

Fig. 4. Regression analysis of a posteriori probability of membership in the normal group versus weeks after diagnosis

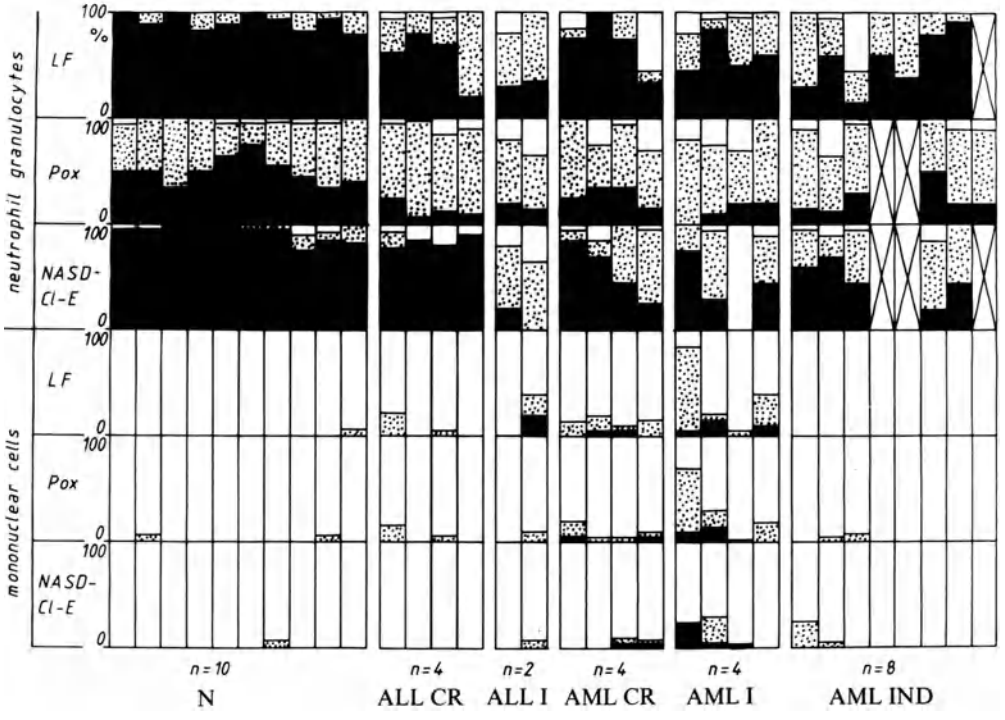

Fig. 5. Peroxidase, NASD-chloroacetate esterase, and lactoferrin staining of peripheral blood neutrophilic granulocytes and mononuclear cells in adult patients with ALL and AML during complete remission at diagnosis and during induction treatment and in adult control. *N,* normal controls; *ALL CR,* ALL in complete remission; *ALL I,* ALL at diagnosis; *AML CR,* AML in complete remission; *AML I,* AML at diagnosis; *AML IND,* AML induction chemotherapy; *LF,* lactoferrin; *Pox,* Peroxidase; *NASD-Cl-E,* NASD-chloroacetate esterase; ■, + + cells; ⊡, + – cells; □, – cells

analysis of peripheral blood cells was carried out. Figure 3 shows the results of MPO staining for PMN in children with ALL in complete remission off chemotherapy and on maintenance treatment, and for comparison the juvenile control group. In our search also for a more sensitive marker for granulopoietic differentiation and proliferation we looked for intracellular lactoferrin in PMN as well as MPO and myeloesterases (Fig. 5).

The observations in adult patients yielded very similar results to those seen in children, demonstrating deficient enzyme activities and lactoferrin staining in acute leukemia on and off therapy. Cytochemical stains for myeloid NASD-chloroacetate esterase yielded similar results to those obtained by MPO staining as well as immunofluorescence and immunoperoxidase staining for lactoferrin.

The results of these studies support the hypothesis that bone marrow function of patients with acute leukemia does not become completely normal even in clinical long-term complete remission.

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Studies on Normal B-Cells and Common Acute Lymphoblastic Leukemia Blast Cells Using a Colony Assay

C. A. Izaguirre and M. F. Greaves

Human B-cell populations contain a subset of cells that are capable of forming colonies in culture [1]. This in vitro assay permits the study of growth and differentiation of the clonogenic cells both in normal and various malignant diseases of the B-cell lineage. It is also applicable to common acute lymphoblastic leukaemia (cALL) [2] and B-cell chronic lymphocytic leukaemia [3]. The specificity of the assay depends on exhaustive depletion of T-cells from B-cell containing populations because the conditioned medium used in the assay (PHA-TCM) also contains factors that promote T-cell colony formation. PHA-TCM is prepared from purified T-cells cultured in the presence of 1% phytohaemaglutinin for 3 days. Finally, normal T cells must be included as feeder cells in the culture mixture, after treatment with mitomycin-C or radiation; myeloma cells are an exception as they do not require feeder cells [1, 4]. Methyl cellulose is used as viscous support medium; alpha-MEM or Iscove's medium (GIBCO) and fetal calf serum complete the culture mixture. Colonies are scored after 7 days of culture.

In this paper we present preliminary data on some of the characteristics of the cells that give rise to B-cell and common ALL colonies.

Blood cells were obtained from normal volunteers and separated using density gradients, sheep red cell rosetting and adherent cell depletion techniques as previously described [1]. A T-cell rich fraction (E+ cells) and a B-cell rich fraction (E^- cells) depleted of T cells (<2%) and adherent cells were obtained.

The E+ cells were used as source of feeder T cells and to prepare PHA-TCM. The B-cell rich fraction contained 20%–50% B cells. This fraction was further separated using the fluorescent-activated cell sorter (FACS 1) and two markers specific for B-lymphocytes, a polyvalent goat anti-human immunoglobulin conjugated with fluorescein to label surface immunoglobulin-(SIg) positive B cells and a monoclonal antibody (B1) that reacts with all blood B-lymphocytes [5] (Coulter Electronics Ltd, Hialeah, Florida). The monoclonal antibody was developed with a goat anti-mouse immunoglobulin fluorescein-conjugated.

Cells from the unsorted fraction, the purified B-lymphocytes (sIg+ or B1+ cells) and a B-cell depleted (sIg- or B1- cells) fraction were cultured using the B-cell colony assay. After 7 days in culture, the colonies were counted and then analysed for cell markers. First, the cytoplasm of cells from individual colonies from the sIg+ cell fraction were simultaneously stained with a mixture of a goat anti-human kappa rhodamine-conjugated and goat anti-human lambda fluorescein-conjugated. Each colony was scored twice for red or green fluorescence using fluorescence microscopy. In a first experiment 17 of 22 colonies were kappa (+) only and 2 of 22 colonies were lambda (+) only. In a second experiment 14 of 24 colonies were kappa (+) only and 8 of 24 were lambda (+) only. These results suggest that each colony derives from a single cell B-cell precursor, therefore fulfilling an important requisite for a clonogenic assay.

Table 1. Monoclonal antibodies

Antibody	Specifity	Source	Reference
J5	Common ALL antigen	Coulter	[12]
B1	B-cell specific	Coulter	[5]
UCHT-2	Pan-T cell	P Beverley	[7]
UCHT-1	Mature T cell	P Beverley	[8]
OKT11	Sheep Red Cell Receptor	Ortho	[6]
Leu 2a	Suppressor Cycotoxic T cells	Beckton-Dickinson	[11]
Leu 3a	Helper-Inducer T cells	Beckton-Dickinson	[11]
Leu 7	NK cells	Beckton-Dickinson	[9]
OKM1	NK cells, monocytes	Ortho	[10]

Table 2. Fluorescent-activated cell sorter analysis of B-cell colony forming cells

Antibody	Experiment no.	Cell sorter Fraction	No. of colonies per 2×10^4 cells	Colonial cell phenotype
Surface				
Ig	1	Control	237	n.t.[a]
(polyvalent)		Negative	102	n.t.
		Positive	332	*B cells only*[b]
	2	Control	130	n.t.
		Negative	51	n.t.
		Positive	215	*B cells only*[b]
B1	1	Control	107	B + T cells
(monoclonal)		Negative	157	T cells only
		Positive	99	B cells only
	2	Control	192	B + T cells
		Negative	162	T cells only
		Positive	115	*B cells*

[a] n.t., not tested
[b] Used to determine kappa and lambda distribution in single colonies

Secondly, colonies from each fraction were collected, pooled, and a single cell suspension was prepared and stained with B- and T-cell markers (Table 1). The results shown in Table 2 reveal that pure B-cell colonies are obtained only in positively purified B-cell fractions (sIg+, B1+), indicating that the B-cell colony forming cell in blood has a mature B-cell phenotype with sIg and the B1 antigen on its surface. The table also shows that T-cell growth occurs in unsorted E^- cells, suggesting that this level of cell separation in normal blood is not sufficient to obtain pure B-cell colonies. The colonies obtained from the B-cell depleted B1(–) cells do not react with B-cell markers but react with some pan-T cell markers, OKT11 (94%) and UCHT-2 (70%) [6, 7], but not with a mature T-cell marker, UCHT-1 (3%) [8], suggesting that these colonies contain cells belonging to a different T-cell subset. Markers of NK cells (natural killers) are found amongst these cells, Leu 7 (8%) [9], and OKM1 (24%) [10] as well as markers of helper T cells [11], Leu 3a (48%). No Leu 2a (suppressor) [12] positive cells were detected. Further analysis is required to confirm these findings and to determine if these T-cell colonies derived from contaminating T-cells or from T-cell marker negative cells.

Blood cells from patients with common ALL were depleted of T cells as described above [2] and then further separated in the cell sorter using a monoclonal antibody (J5) against the cALL antigen. cALL-positive (J5+) and cALL-negative (J5–) fractions were collected. In two experiments

the unsorted, the J5– and the J5+ fractions all gave rise to cALL colonies (Experiment 1: 295, 201 and 235 colonies per 4×10^4 cells; Experiment 2: 105, 96 and 126 colonies per 4×10^4 cells).

In summary, (a) B-cell colonies from normal blood cells arise from single cells that have a mature B-cell phenotype: surface Ig+, B1 positive; (b) the common ALL antigen is not a marker of all common ALL clonogenic cells, and (c) T-cell colonies arise from B- and T-cell depleted fractions; they may belong to a subset of T-cells that includes NK cells.

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Human Interleukin 2: Physiology, Biochemistry, and Pathophysiology in Lymphoblastic Leukemias and Immunodeficiency Syndromes*

K. Welte, S. Venuta, C. Y. Wang, S. P. Feldman, N. Ciobanu, G. Kruger, H. J. Feickert, V. J. Merluzzi, N. Flomenberg, M. A. S. Moore, and R. Mertelsmann

A. Introduction

The regulation of immune function [6] and tumor growth [16] by hormone-like factors, cytokines, has become the subject of increasing interest. Interleukin 2 (IL2) discovered by Morgan et al. [5], is produced by T-lymphocytes after antigen or mitogen stimulation and is required for the proliferation of activated T cells. IL2 is an essential mediator of the immune response [11, 15], and there is preliminary evidence that it may also be responsible for the clonal growth of human lymphoblastic leukemias [17].

Studies on the physiology and pathophysiology of IL2 are dependent on the availability of a well-defined, biochemically, and biological homogeneous molecule. We have therefore purified IL2 to apparent homogeneity [18] and have started to examine its role as mediator of the normal immune response, in human immunodeficiency syndromes and in acute lymphoblastic leukemias (ALL).

B. Methodology

Heparinized blood samples were drawn from healthy volunteers and patients after obtaining informed consent. Ficoll-Hypaque separated mononuclear blood cells were resuspended at 4×10^6 cells/ml in RPMI 1640 supplemented with 5% heat-inactivated FCS and glutamine (2 m*M*). Each sample was stimulated in triplicate microwell cultures (# 3596 culture plate, Costar Inc. Cambridge, MA) with one of the following: (a) medium alone, (b) phytohemagglutinin (PHA-M, 0.5% by volume, Grand Island Biological CO), (c) OKT3 (Ortho Diagnostic Systems, Inc., Raritan, NJ) or (d) Pan T2 (Wang et al. 1982, submitted).

C. Results

I. Physiology of lymphocyte proliferation and IL2 Production Induced by PHA and Mitogenic Antibodies

Stimulation assays were done with or without the addition of irradiated Daudi cells (5000 rads) at a final concentration of 0.5×10^6 cells/ml. At indicated time points 100 µl supernatants were removed from each well to be assayed for IL2. Identical cultures were pulsed for 4 h with tritiated thymidine [^{3}H]dT (0.5 µci/microplate well, specific activity 20 mci/m*M*. New England Nuclear, Boston, MA) and the incorporation of [^{3}H]dT measured.

The IL2 microassay, definition of units, and biochemical techniques have been published in detail elsewhere [1, 18].

1. Mitogenesis Induced by PHA, Pan T2, and OKT3

PHA as well as both T-cell specific antibodies were able to induce a proliferative response in normal PBL incubated for 4

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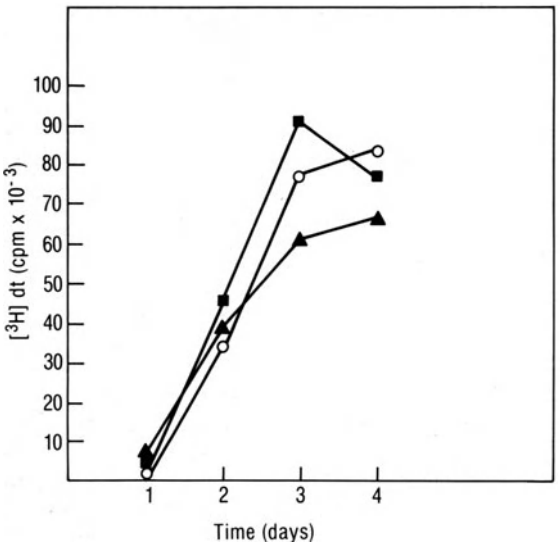

Fig. 1. Mitogenic effect of Pan T2, OKT3 and PHA on normal PBL. PBL were plated in 96-well microtiter plates at 4×10^6/ml in RPMI supplemented with 5% heat- inactivated FCS and Pan T2 (500 ng/ml), OKT3 (1.25 ng/ml), or PHA (0.5%). For each of 4 days thereafter, 0.5 μCi [^{3}H]dT was added to measure DNA snthesis. Each *point* is the average of a triplicate determination. ■, PHA; ○, Pan T2; ▲, OKT3

days a measured by incorporation of tritiated thymidine (Fig. 1). There was no significant difference between the two antibodies when used at saturating concentrations. However, the amount of Pan T2 (10^{-10} *M*) needed to induce maximum mitogenesis was 100 times more than the concentration required for OKT3 (10^{-12} *M*).

In the presence of irradiated Daudi cells we observed a twofold increase in cell proliferation with Pan T2. In contrast, the costimulation with Daudi cells on OKT3 had essentially no effect.

2. IL2 Production Induced by Monoclonal Antibodies

Pan T2 and OKT3 induced relatively low levels of IL2 production. However, when these cells were incubated in the presence of each antibody plus Daudi cells the amount of IL2 induced by Pan T2 was

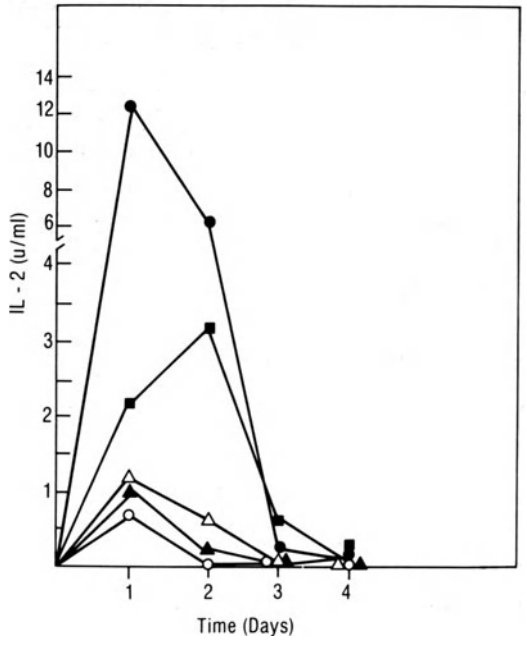

Fig. 2. IL2 production induced by Pan T2 and OKT3 on normal PBL in the presence or absence of Daudi cells. Irradiated Dudi cells were plated at 0.5×10^5/ml, where indicated. PHA (0.5%) was added to the control sample. IL2 concentration was determined in the harvested culture medium as described. ●, Pan T2 + Daudi; ■, PHA; △, OKT3 + Daudi; ▲, OKT3; ○, Pan T2

Table 1. IL 2 production by normal lymphocytes (PBL) and leukemic lymphoblasts (ALL) in response to PHA and T-cell monoclonal antibodies with and without costimulators

	PBL (U/ml)	ALL-4 (U/ml)
Media	0	0
PHA	1.2	1.4
Pan T2	1.2	0
Pan T2 + Daudi	12.5	3.0
Pan T2 + Protein A	1.5	0
OKT 3	2.1	0.8
OKT 3 + Daudi	22.2	0.9
OKT 3 + Protein A	8.5	1.6

more than tenfold higher compared with OKT3 (Fig. 2).

3. Effect of protein A on IL-2 Production Induced by Pan T2 and OKT3

Protein A (40 μg/ml) was added to the microwell cell supensions of normal PBL in the presene of either Pan T2 or OKT3. As shown in Table 1, protein A had only a negligible effect on the IL-2 production induced by Pan T2, while it enhanced the stimulation by OKT3 approximately fourfold. (In contrast, irradiated Daudi cells are potent costimulators for the Pan T2 response while they are without effect on the OKT3 response).

4. Inhibition of IL2 Production and Response

Further studies also suggest a role for HLA-DR antigens in the regulation of the IL2 production [7]. The extent of inhibition by antibodies against these structures is dependent on the mitogen used (own observation), suggesting that Pan T2 and OKT3 bind to different subunits of the T-cell activation antigen recognition complex. This has recently been continued through immunoprecipitation studies (Wang et al. 1982, submitted).

5. Stimulation of PBL Proliferation by Anti M7 and Anti RD 114 Antiserum

Table 2 shows that goat antisera raised against the baboon endogenous virus, M7, or RD114 were able to stimulate the proliferation of PBL, while goat anti-simian sarcoma virus (SSV) antiserum was unable to do so. Absorption of the antisera with M7 or RD114 virus removed the sera capacity to induce cell proliferation. These data suggest a common antigenic determinant shared by the T-cell activation/antigen recognition complex and M7 as well as RD 114.

II. Biochemistry

The purification of IL-2 from lymphocyte-conditioned medium (Ly-CM) has been reported in detail elsewhere [18]. Briefly, IL2, produced with or without costimulation by irradiated cells of the Burkitt's lymphoma line Daudi, was purified 37,000-fold to apparent homogeneity from Ly-CM by sequential $(NH_4)_2SO_4$-precipitation, ion exchange chromatography (DEAE-cellulose), gel filtration, and chromatography on

Table 2. Proliferation of PBL after stimulation with goat anti-virus sera

Stimulus	Proliferation [^{3}H]dT incorporation (cpm)
None	2,800
Pan T2	47,000
Anti-M7	35,000
Anti-RD114	41,500
Anti-M7 absorbed with M7	15,000
Anti-RD114 absorbed with RD114	7,800
Anti-SSV	2,700

Antisera were used at 1:125 dilutions. For absorption 1 ml of antiserum was incubated overnight at 20 °C with 1.5 mg of virus. All other conditions were identical to those in Fig. 1. [^{3}H]dT incorporation was measured 2 days after stimulation

Table 3. Biochemical characteristics of IL2 produced by PBL and leukemic lymphoblasts (ALL) in the presence and absence of Daudi cells

Method	*PBL(+Daudi)*	*PBL(–Daudi)*	*ALL(+/–Daudi)*
Molecular weight (AcA 54 Ultrogel filtration)	14,000	26,000	26,000
Molecular weight (SDS-PAGE, reduced conditions)	14,500	16,000 and 17,000	16,000 and 17,000
Isoelectric point	8.1	6.7	6.6
Hydrophobic binding to Blue agarose and Procion-red agarose	Strong	Strong	Strong
Glycoprotein	No evidence	No evidence	No evidence
pH stability	2 – 10	2 – 10	2 – 10

blue agarose and on Procion-red agarose. The purified IL2 showed a specific activity of 10^6 U/mg protein. IL2 produced in the absence of Daudi cells exhibited a native molecular weight of 26,000 as measured by gel filtration and an isoelectric point of 6.7. This IL-2 showed 16,000 and 17,000 mol wt. bands in SDS-polyacryladmide gel electrophoresis. IL-2, produced in the presence of Daudi cells, showed a molecular weight of 14,000, as measured by both gel filtration and SDS-polyacrylamide gel electrophoresis, and an isoelectric point of 8.1 (Table 3). The purified IL-2 lacked detectable activities of all cytokines tested: interferon (α and γ), granulocyte-macrophage-colony stimulating factor, B-cell growth factor, T-cell replacing factor, B-cell differentiation factor, macrophage activation factor, and thymocyte-differentiating activity. It was free of any contaminating proteins as judged by silver staining in SDS-polyacrylamide gel electrophoresis. All three molecular forms of IL-2 were biologically active, supporting the growth of human and murine cytotoxic T-cell lines at concentrations of 10^{-11}–10^{-10} *M*.

III. Antibody Aainst IL2

We used the purified IL2 for the production of a mouse monoclonal antibody against IL2. The fusion resulted in hybrid clones producing anti-IL2 of various subclasses (IgA, IgG-2b, IgM). All anti-IL2 antibodies inhibited the proliferation of IL2-dependent human and mouse cell lines in reponse to human highly purified IL2. One of these antibodies chosen for further characterization precipitated $14K^{125}$I-IL2 as well as $16K^{125}$I-IL2 and $17K^{125}$I-IL2 (Feickert et al. 1982, submitted).

IV. IL2 Production by Fresh Lymphoblastic Cells and the Lymphoblastic Cell Line JM[1]

1. Production of IL2 by Leukemic Cells

Leukemic cells were cultured in the presence of PHA, OKT3, or Pan T2, and tested for IL-2 production and proliferation as described for PBL. IL-2 production induced by PHA and OKT3 stimulation continued to increase over 3 days (Fig. 3) and was not followed by a rise in cell proliferation (not shown). This was in marked contrast to the reponse of PBL to PHA and OKT3 stimulation (Fig. 2). This pattern of response was common to ALL with different phenotypes. Pan T2 was unable to induce either proliferation or IL2 production in ALL. This pattern of response was also markedly different to Pan T2 stimulation of normal PBL.

[1] The cell line Jurkat used by other investigators is a subclone of the original line JM developed by Schneider et al. [13]. Our studies have failed to show any difference between the original JM and the subclone Jurkat and therefore consider the original designation JM more appropriate

JM, a cell line derived from a T-cell ALL, is TdT+, Ia-, E+, Leu I+ and, after PHA stimulation, produces IL2 but does not proliferate. We studied the effect of Pan T2 on this line and found that this monoclonal antibody does not induce IL2 production or stimulate cell proliferation. Therefore, JM and fresh ALL cells have the same pattern of IL2 production and proliferation after PHA or Pan T2 stimulation (Table 1).

The addition of Daudi cells was able to rescue the response of ALL cells to Pan T2, and induced IL2 production. We could not detect any effect of Daudi alone on IL2 production by any of the ALL cells (Table 1) and JM (not shown).

2. *The Factor Produced by the Leukemic Population is IL2*

In order to show that the factor produced by the leukemic cell populations was indeed IL2, we tested if the factor produced by ALL cells was able to support the growth of the human cytotoxic cell line, C13.3 (kindly provided by Dr. N. Flomenberg, Sloan-Kettering Institute), which requires IL2 for survival and proliferation. The factor produced by ALL and IL2 purified from normal PBL supported the growth of C13.3 equally well.

As shown in Table 3 the biochemical characteristics of IL2 produced by leukemic cells are similar to those of IL2 produced by normal PBL. However, the molecular heterogeneity of IL2 produced by leukemic cells was not influenced by Daudi costimulation in contrast to IL2 generated by normal PBL.

Finally, IL2 produced by ALL cells binds to a monoclonal antibody prepared against rat IL2 (analysis performed by Dr. Gillis, Immunex, Seattle, WA) as well as to our own monoclonal antibody against human IL2 (Welte et al., unpublished).

A colony assay for blast cell progenitors in non-B non-T ALL has recently been described by Izzaguire et al. [3]. ALL cells were cultured in methylcellulose in the presence of Ly-CM and feeder T cells. After 5–7 days the colonies exhibited the common ALL phenotype. To test whether growth was dependent on IL2 we substituted partially purified IL2 (DEAE-cellulose fraction) and purified IL2 for the Ly-CM in the presence and absence of feeder T cells. Preliminary results suggest that partially purified IL2 alone can support the growth of ALL cells; however the highly purified

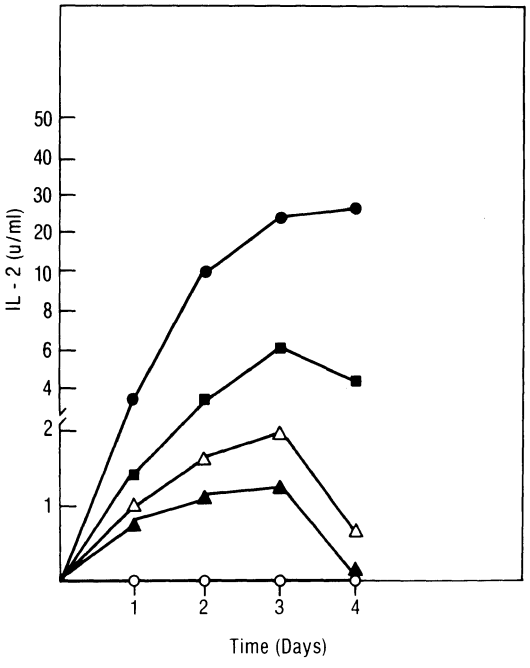

Fig. 3. Effect of Daudi cell costimulation on IL2 production induced by Pan T2 and OKT3 on ALL-4. ALL-4 PBL were plated at 4×10^5 per well. Pan T2 (500 ng/ml) and OKT3 (1.25 ng/ml) were added alone and in the presence of irradiated Daudi cells (0.5×10^5). PHA (0.5%) was added to a control sample. 112 concentration was determined in the harvested culture medium. ■, PHA; ○–○, Pan T2; ▲, OKT3; ●–●, Pan T2 + Daudi; △, OKT3 + Daudi

IL2 reuires feeder T cells for maximum colony formation (Table 4). The cell surface markers of the colonies grown in the presence of purified IL2 exhibited both pre-B and T-cell characteristics. In a more recent experiment with another ALL donor, the majority of the cells were Bl-positive. We are currently testing several additional ALL samples in this assay, and will identify the growth factor requirements of ALL cells in culture to investigate further the hypothesis of autostimulation in ALL.

V. IL2 Production and Proliferation of PBL in Primary and Acquired Immunodeficiency Syndromes (IDS).

1. Mouse model

Spleen cells from mice treated with cyclophosphamide (CY) (150 mg/kg) do not generate effective cytotoxic T-lymphocyte (CTL) responses to allogeneic tumor cells in vitro. When purified human IL2 is added to the culture system, spleen cells from CY-treated mice are able to generate normal CTL responses (Merluzzi et al. 1982, submitted).

2. Combined Varied Immunodeficiency (CVI)

Fifteen patients with CVI and one patient with a related disorder (transient hypogammaglobulinemia of childhood) had a statistically significant decreased response to mitogen stimulation when compared with a control normal population. After addition of purified IL2 the proliferative response was significantly improved with all mitogens used. Two groups could be distinguished: Group A (10/16) had full or partial normalization of proliferative response after addition of IL2, and group B (6/16) had no significant response. One patient showed a decrement in proliferative response after IL2 was added. The results are listed in Table 5 and in Kruger et al. (1982, manuscript submitted).

The production of endogenous IL2 was lower in the group of patients irrespective of the mitogen used when compared with normals (Table 5).

The anti-T-cell monoclonal antibody, Pan T2, recognized a proliferative defect in 5/16 patients which was neither recognized by PHA nor OKT3. This was not significantly correctecd by the addition of IL2. The lack of responsiveness to Pan T2, however, did correlate with the inability of the B cells of these patients to proliferate and differentiate in reponse to B-cell mitogens [12].

Table 4. Growth factor requirements of ALL cells in methylcellulose culture

	IL2 fraction[a]			
	1	3	5	None
Feeder Cells	Colonies/3 × 10^4 cells added			
–	0	308[b]	60	0
+	354[b]	318	200[b]	0
Markers	%			
J5	0	5	2	–
B1	30	10	20	–
T3	0	40	42	–

Colony assays were performed according to Izzaguire et al. [3], with deletions/additions as indicated

[a] 1, Ly-CM; 3, DEAE-cellulose purified IL2; 5, Procion-red agarose purified IL2

[b] Conditions used for marker studies

3. Kaposi's Sarcoma in Acquired Immunodeficiency Syndromes (AID)

Homosexual patients with Kaposi's sarcoma (KS) exhibited a very low proliferative response to OKT3 (15% of the normals). Four out of seven patients also had a very low proliferative response to PHA (10% of the normals) and to Pan T2 (8% of the normals). The production of endogenous IL2 was significantly lower in PBL cultures from KS patients, irrespective of the mitogen used.

Addition of purified IL2 in the presence of these mitogens was able to restore partially or completely the lymphocyte proliferation in all patients tested.

These data suggest that homosexual patients with KS have a defect in IL2 production that is correctable, in vitro, by addition of purified IL2 (Ciobanu et al. 1982, submitted) (Table 5).

Table 5. IL2 production and proliferation (in the absence and presence of 10 U/ml purified IL2) of PBL from patients with common variable immunodeficiencies (CVI), Nezeloff's syndromes, homsexual males with Kaposi's sarcoma, hemophiliacs with acquired IDS, relapsed Hodgkin's disease before retreatment, and healthy controls

Diagnosis (n = number of patients)	PHA			OKT 3			Pan T2		
	IL2 (u/ml)	Prolif. – IL2 (cpm × 10^3)	Prolif. +IL2 (cpm × 10^3)	IL2 (u/ml)	Prolif. – IL2 (cpm × 10^3)	Prolif. +IL2 (cpm × 10^3)	IL2 (u/ml)	Prolif. – IL2 (cpm × 10^3)	Prolif. +IL2 (cpm × 10^3)
CVI, group 1 (IL2 responder) $n = 10$	0.6 (0.3 – 1.7)	43 (29 – 58.6)	78 (39 – 117)	0.5 (0.2 – 1.5)	32 (6.3 – 88.1)	55 (35 – 217)	0.5 (0.3 – 1.1)	16 (1 – 88)	35 (8.2 – 128)
CVI, group 2 (IL2 nonresponder) $n = 6$	0.9 (0.3 – 7.8)	14 (8 – 51)	18 (3 – 58)	0.5 (0.3 – 1.4)	16 (7.6 – 53)	16 (7 – 65)	0.5 (0.4 – 0.6)	8 (0.5 – 21)	7 (0.5 – 23)
Nezeloff's syndrome $n = 2$	0.2	1) 1.8 2) 1.8	1) 12.7 2) 17.2	0.2	1) 1.3 2) 1.6	1) 3.9 2) 22	0.2	1) 1.3 2) 1.6	1) 5.4 2) 16.1
Kaposi's sarcoma $n = 12$	1.1 (0 – 4.2)	20 (1.6 – 72)	28 (15 – 108)	0.9 (0.2 – 1.8)	12 (3.5 – 32)	39 (13 – 80)	0.3 (0 – 1.3)	15 (0.5 – 50)	34 (0.7 – 82)
Hemophiliacs $n = 3$	1.2 (0.7 – 1.8)	12 (4.5 – 44)	18 (7.5 – 42)	0.7 (0.6 – 0.8)	8.7 (1.7 – 10.3)	16 (13 – 25)	0.7 (0 – 0.8)	0.5 (0.2 – 12)	9 (0.6 – 16)
Hodgkin's disease $n = 7$	n.t.[a]	5 (1 – 48)	13 (3 – 73)	n.t.	4 (1 – 50)	17 (3 – 83)	n.t.	2.6 (0.5.4)	14 (3 – 22)
Control $n = 20$	2.3 (0.8 – 6.8)	80 (56 – 110)	90 (52 – 127)	3.5 (0.9 – 16)	61 (37 – 88)	82 (51 – 127)	0.7 (0.4 – 24)	59 (22 – 88)	84 (50 – 125)

Results are shown as medians and ranges in parenthesis; IL2 was measured at day 1, proliferation at day 3

[a] n.t., not tested

4. Hodgkin's Disease

All patients with relapsed Hodgkin's disease before retreatment showed a decreased T-cell proliferative response to all mitogens used and had a partial normalization of T-cell proliferative response after addition of purified 112 (Table 5).

5. Hemophiliacs with Acquired Immunodeficiency

Three of five patients with hemophilia examined had an abnormal T-cell proliferation pattern purified with a partial normalization in the presence of IL2 (Bussel et al. manuscript in preparation) (Table 5).

6. Primary Immunodeficiency Syndromes

One child with Nezelof's syndrome showed in vitro restoration by purified IL2 of the proliferation in reponse to alloantigens and mitogens. After 6-day MLC in the presence of IL2, effector cells capable of NK and alloreactive cytotoxicity against PHA lymphoblasts and neoplastic cell lines were recovered. No viable cells were recovered from similar in vitro cultures in the absence of IL2. A second Nezelof's patient showed augmentation of his NK activity but no restoration of the allocytotoxic reponse. The proliferative response to PHA, OKT3, and Pan T2 is shown in Table 5. Patient 1, who demonstrated a positive in vitro response to IL2, was subsequently given IL2 subcutaneously as part of a recently initiated phase I trial. Though the patient died several days after the trial was begun due to pulmonary infection, examination of his lymphoid tissues postmortem suggested that purified IL2 may have exerted an in vivo effect on his T-lymphocytes. At autopsy, his lymphoid tissues showed only histocytes and plasma cells except in the lymph nodes draining the IL2 administration sites, where nests of lymphoid cells were identified.

These studies suggest that some primary and acquired immunodeficiencies may be caused by defects in IL2 production and/or response. In addition, they have provided some preliminary evidence that highly purified IL2 is capable of producing an in vivo effect in appropriate immunodeficient patients (Flomenberg et al. 1982, submitted).

D. Discussion

I. Physiology of IL2 Production and Response

The introduction of T-lymphocyte specific monoclonal antibodies has facilitated the ability to comprehend further the complex interaction and control of the immune response. The binding of the antibodies Pan T2 and OKT3 to specific antigenic determinants (T-cell activation/antigen recognition complex) on the surface of T-lymphocytes is able to trigger a proliferative response similar to antigenic stimulation or mitogensis. It has been observed that OKT3 is mitogenic even in the range of 10^{-12} *M*, while Pan T2 is less potent, requiring a concentration of 10^{-10} *M* for maximum stimulation. While highly costimulatory with Pan T2, Daudi cells had no significant effect on either IL2 production or cell proliferation in the presence of OKT3. Daudi cells have been used by several investigators to enhance IL2 production from normal PBL [2, 10, 11, 18]. The effect of Daudi cells could be mediated by (a) Ia antigen, (b) Fc receptors, and (c) an additional effector molecule. Both Ia antigen [7] and Fc receptors [14] have been implicated in the augmentation of IL2 production.

II. Biochemistry

The purification steps described in this study produced IL2 with a specific activity of 10^6 U/mg protein. Because the lowest molecular weight of an active IL2 polypeptide was 14,000, it could be calculated that IU/ml of IL2 was equivalent to a molar concentration of 7×10^{-11} *M*. An IL2 concentration of 1.4×10^{-11} *M*, or 4×10^5 molecules/cell, was required for one-half maximum stimulation of murine CTLL. All other purification methods [2, 4] have achieved neither a specific activity nor a yield comparable to those described here.

Native IL2 has been previously shown to exist in several molecular forms. Here, we show that the stimuli used for IL2 induction by PBL can be responsible for this heterogeneity. IL2 produced in the presence or absence of Daudi cells had a molecular weight of 14,000 and 26,000, respectively, by gel filtration and 14,500 and 16,000–17,000 repsectively by SDS-polyacrylamide electrophoresis. All molecular forms could be obtained by varying the concentration of costimulator cells.

The effect of Daudi cells on the IL2 production, however, does appear to be complicated in view of (a) the shift in molecular weight of IL2 induced by Daudi cells in PBL, and (b) the superinduction of IL2 in PBL and in human lymphoblastic leukemic cells by costimulation with Daudi cells. The possibility that different T-cell subsets or different leukemic phenotypes are responsible for the production of the two IL2 forms is currently under investigation.

III. IL2 in Lymphoblastic Leukemias

There is evidence that the growth of at least some human malignant cells is factor dependent and that the malignant cells are capable of producing these factors ("autostimulation," [16]).

We have studied the capacity of leukemic cells to produce and respond to IL2. The leukemic cells studied were either non-T, non-B ALL, or T-cell ALL. In every case, the cells produced a large quantity of IL2. This factor had physicochemical characteristics identical to that of normal IL2, with a mol. wt. of 16,000–17,000 and pI of 6.6 (Table 3) and reacted with monoclonal antibodies directed against normal IL2. These data therefore strongly support that the factor produced by ALL cells is identical or at least closely related to IL2. Costimulation of ALL cells by PHA and Daudi cell, however, failed to lead to a shift in molecular weight, suggesting a restricted expression of IL2 species in ALL. Further studies performed argue against the possibility that residual normal T cells are responsible for the IL2 production by extensive cell purification techniques (repeated E-rosetting, density gradient centrifugation of hypodiploid or hyperdiploid leukemic cells). The leukemic population studied could not have had more than 1% normal cells based on flowcytometric analysis of DNA ploidy levels.

While OKT3 was able to induce IL2 production from leukemic cells, Pan T2 alone was unable to cause the release of IL2 from ALL cells. However, the Pan T2 activation "pathway" is not completely repressed in ALL cells since it can be activated by costimulating ALL cells with Pan T2 and Daudi. The pattern of response of ALL cells to Pan T2 and Daudi suggests that the IL2 producer cell in the ALL population has an altered Pan T2 receptor complex, which could play a role in leukemogenesis.

This conclusion is supported by the study of JM, a leukemic T-cell line. The characteristics of IL2 production in this clonal population of leukemic cells were found to be similar to those of ALL cells.

How Daudi cells are able to restore the ability of Pan T2 to induce IL2 may be important in further understanding the lack of normal control mechanisms on cell proliferation in ALL.

The role of IL2 production in ALL remains to be determined. It appears unlikely that the release of this factor, critical for the proliferation of cells of T lineage, is only an epiphenomenon in ALL, irrelevant for the expansion of the leukemic clone. Recently, a clonal assay system permitting the growth of blast cell progenitors in non- T, non-B ALL has been developed [3]. In this assay, factors present in Ly-CM are required for the successful growth of the leukemic stem cells. Since (a) IL2 is present in Ly-CM, (b) IL2 produced by leukemic and normal T cells appears to be identical (Table 3), (c) peripheral blast populations of ALL do not proliferate in response to IL2, as measured by [^{3}H]dT incorporation, we hypothesize that IL2 is a factor (or one of the factors) produced by partially differentiated leukemic cells and required for the replication of the leukemic stem cells. Since leukemic stem cells represent only a small percentage of the total leukemic population, its proliferation cannot be shown in the assays used for normal PBL. The clonal assay should be able to clarify this important point. Our preliminary data suggest that highly purified IL2 is able to substitute for Ly-CM (Feldman, Izzaguire, Mertelsmann, unpublished data).

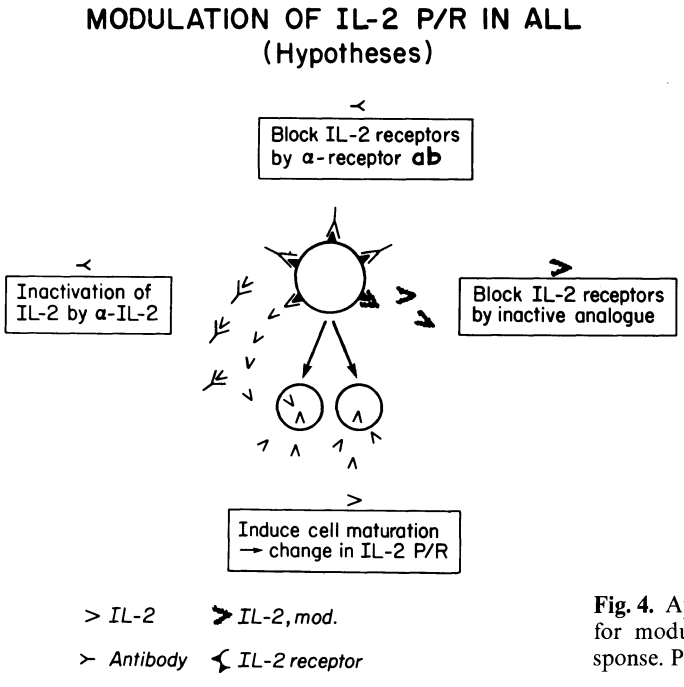

Fig. 4. Approaches under investigation for modulating IL2 production and response. P/R = production/response

In this context, it is of great interest that neoplastic T cells from patients with cutaneous T-cell lymphoma (CTCL) grow in medium containing partially purified IL2 without prior stimulation. Neoplastic T cells differ therefore from normal T cells in not requiring in vitro activation by lectin to interact with IL2 [8], suggesting induction of IL2 receptors by the HTLV [9]. Still another possibility is that leukemic stem cells have a specific way of turning on the release of IL2 (or other factors) from the producer cell. We have shown that normal cells have receptors for OKT3 and Pan T2 which induce IL2 production and cell proliferation (Feldman et al. 1982, submitted). We have further demonstrated that the receptor for Pan T2 is altered in ALL cells [17]. These data strongly suggest a very specific alteration of the membrane of ALL cells.

The importance of the molecular structures present on the surface of T cell for growth regulation is also shown by our preliminary studies of sera raised against endogenous viruses. These sera directed against baboon endogenous virus, M7, and the closely related virus RD114 stimulated the proliferation of normal PBL (Table 2) and the release of IL2 (not shown). Serum raised against SSV was unable to do so. These results also indicate that endogenous virus interacts with growth regulator sites present on the T-cell surface and should be important for the understanding of leukemogenesis and autoimmune diseases.

Modulation of IL2 production and response in vivo could provide a new and powerful approach to manipulate proliferation of specific normal and neoplastic cells in vivo. Figure 4 illustrates several possibilities of manipulating IL2 physiology. Studies of both the effect of the anti-IL2 antibody as well of cytotoxic agents coupled to IL2 have been initiated.

IV. IL2 in Immunodeficiency Syndromes

It is well known that IL2 plays an important role in the development of a variety of T-cell responses. We suggest that some human disorders associated with T-cell defects might be due to defective IL2 production or response. We have recently begun to investigate the role of IL2 in primary and acquired immunodeficiency syndromes. The data obtained so far show complete or partial normalization of T-cell

proliferation by purified IL2 in vitro in the majority of patients with Kaposi sarcoma, CVI, Hodgkin's disease, hemophiliacs with acquired IDS, chemotherapy-induced immunosupression (data not shown), and burn patients (data not shown). Since these observations suggest an important in vivo role of IL2 in several congenital and acquired IDS, we have initiated a phase I clinical trial of IL2. The preliminary results support in vivo activity of subcutaneously administered IL2, both in animal models (Merluzzi et al., unpublished) and in man (Flomenberg et al., unpublished).

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Studies of Myelopoiesis Using Monoclonal Antibodies and Variant Lines from the Promyeloid Cell Line HL60*

G. Brown, A. G. Fisher, C. M. Bunce, P. C. W. Stone, and D. Toksoz

Surface marker studies of human leukaemia cells argue in favour of a normal cell surface phenotype and that unique surface changes are not essential to malignancy. This has led to speculation that leukaemia may result "from a subtle uncoupling of the controls which integrate proliferation with maturation" [1]. Studies using in vitro assays for haemopoietic progenitor cells (for example, GM-CFUc assay) have shown that specific humoral factors regulate normal haemopoiesis (GM-CSFs). Thus, the process of differentiation can in part be explained in terms of the expression of discriminatory surface receptors which are essential to alter (or maintain) the maturation status of cells. However, in order to investigate the underlying set of intracellular regulatory mechanisms which integrate proliferation with maturation it will be necessary to (a) work with cloned populations of cells and (b) have convenient markers for structures which are essential to cell proliferation and maturation within a particular lineage.

Various haemopoietic cell lines, which can be induced to differentiate, are available and can be used to study the process of differentiation. The continuous human promyeloid cell line HL60 can be induced to differentiate into neutrophils by 1.25% dimethylsulphoxide (DMSO) and related compounds [2]. When treated with 12-0-tetradecanoylphorbol-13-acetate (TPA) [3] or T-lymphocyte conditioned medium [4] the HL60 cells mature into monocytes/macrophages. The bipotential nature of this cell line may also provide insight to the processes which ultimately restrict the differentiation capacities of cells to a particular lineage.

Monoclonal antibodies to myeloid-associated surface antigens are convenient markers for the differentiated properties and the "maturation programme" of myeloid cells. The availability of the HL60 line provides the means of readily obtaining large numbers of early myeloid cells necessary for raising and characterising monoclonal antibodies. Hybridomas were produced using mice immunised with HL60 cells coated with antibody raised in mice against normal peripheral blood leucocytes [5]. After immunising with coated cells, 7 out of 22 (32%) hybridomas produced antibodies which identify antigens selectively expressed by myeloid cells. Six of these hybridomas have been cloned and the specificities of the antibodies AGF4.36 and 4.48 have been described in detail previously [5].

The distribution of myeloid antigens at different stages of differentiation was investigated using autoradiographs on bone marrow and peripheral blood cells. The results are shown in Fig. 1. Five of the antibodies (AGF4.48, 8.19, 8.29, 8.41 and 9.47) identify an antigen(s) expressed at the promyeloid to blood neutrophil stages of myeloid maturation. In contrast, one of the antibodies (AGF4.36) defines an antigen which is transiently expressed on cells at the promyeloid to metamyeloid stages and is absent from most bone marrow and blood neutrophils. Loss of the AGF4.36 antigen during differentiation was also observed when HL60 cells were induced to

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mature using 1.25% DMSO. The disappearance of the AGF4.36 antigen was compared with the loss of the BK19.9 antigen, which is a marker for proliferating cells and identifies the transferrin receptor [6]. As shown in Fig. 1, the decrease in the number of AGF4.36 antigen-positive cells (from 87% to 37%) correlates with the maturation, as opposed to proliferation of the HL60 cells. The above panel of antibodies identify differentiated properties of neutrophils. In particular, the transient expression of the AGF4.36 antigen suggests that this antibody may identify a membrane change pertinent to differentiation.

The regulation of the expression of the aforementioned surface properties of HL60 cells can be revealed through its alteration and the use of cloned variant lines from HL60. Seven variants of the HL60 line have been isolated which do not mature in response to 1.25% DMSO. These lines were isolated, in medium containing 1.25% DMSO, after culturing the HL60 cells in the presence of SV40 virus (HL60m2 and m4) [7], treating HL60 cells with alpha (α) particle radiation (HL60 Ast1, 3, 4 and 25) or from untreated HL60 cultures (HL60Sp1). The variant cells are morphologically and cytochemically similar to HL60 and are able to differentiate along the monocyte series when treated with TPA [7]. The expression of myeloid antigens by the variant lines is shown in Fig. 2. In contrast to HL60, four of these lines fail to express the 'transient' myeloid antigen (AGF4.36) and show a reduced expression of myeloid antigens. HLA class 1 (2A1), a leucocyte-associated antigen (BK19.45) and the transferrin receptor (BK19.9) are expressed in similar amounts on the HL60 and variant cell lines. The lines Ast3, 4 and Sp1, which express myeloid antigens to be same extent as HL60, indicate that the absence or reduced expression of myeloid antigens is not an artefact of prolonged growth in DMSO. These lines are currently being karyotyped with a view to identifying chromosomal aberrations relating to the above changes.

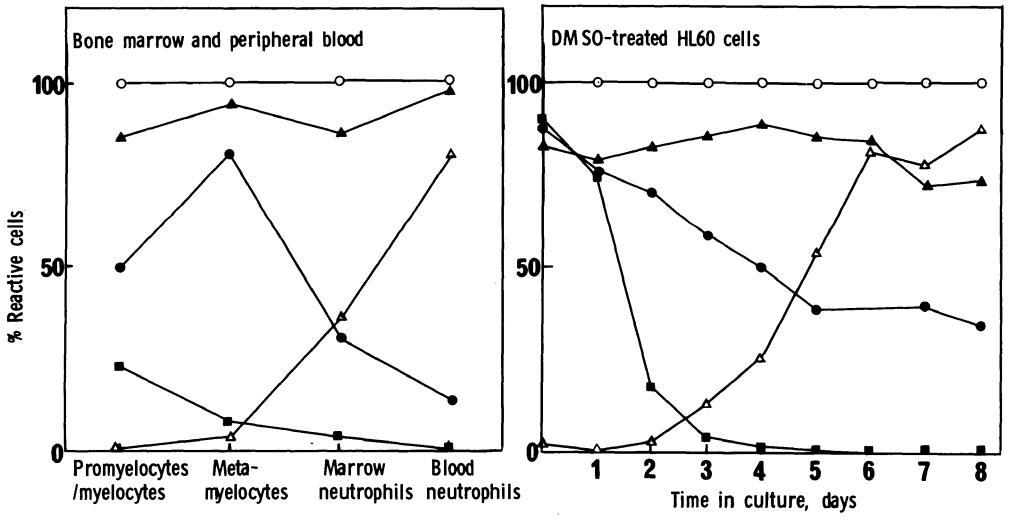

Fig. 1. The distribution of various antigens at different stages of myeloid maturation. The labelling by antibodies of marrow and peripheral blood cells was determined by autoradiography. Results are expressed as a percentage of morphological cell type. Reactivity of the antibodies with DMSO-treated HL60 cells was determined by indirect immunofluorescence. ●—●, AGF4.36 (anti-myeloid); ▲—▲, AGF4.48, 8.19, 8.29, 8.41 (anti-myeloid); ■—■, BK19.9 (anti-transferrin receptor); ○—○, BK19.45 (reacts with leucocytes only). The reactivity of antibody AGF9.47 against marrow and blood cells is similar to ▲—▲ except only 62% of blood neutrophils were reactive. Erythroid precursors were unreactive with the AGF series and monocytes (<1%–46%) were weakly stained. The percentage of antigen-positive cells is compared with the Fc-mediated phagocytosis of marrow and blood cells (△—△) (8) and the ability of the HL60 cells to phagocytose complement-coated yeast cells (△—△).

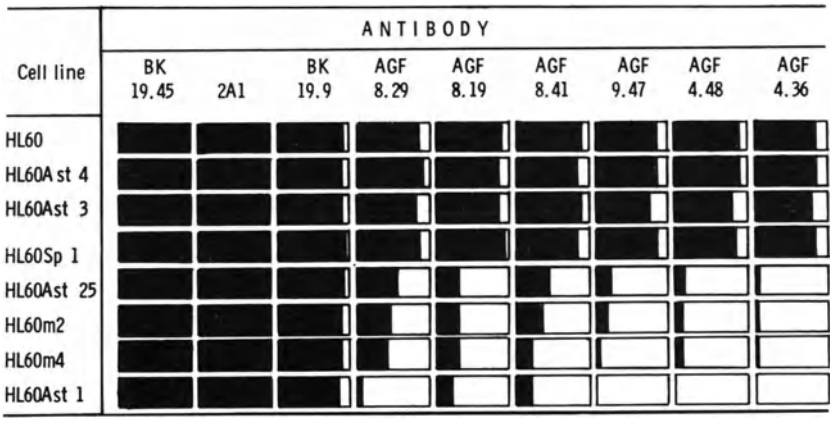

Fig. 2. Expression of surface antigens by variant cell lines from the human promyelocyte line HL60. The *shaded areas* indicate the proportion of reactive cells as determined by indirect immunofluorescence. The antibodies are described in the legend to Fig. 1, with the exception of 2A1 which reacts with class 1 HLA antigens.

The variant lines HL60m2, m4 and Ast25 can be induced to differentiate into neutrophils using higher DMSO concentrations (1.5%–1.75%). In the case of HL60m2 and m4, up to 70% of the cells matured and during this process failed to express the transient myeloid antigen (AGF4.36) and retained reduced amounts of the myeloid antigen (AGF4.48) [5]. The Ast 1 line, which is most affected in terms of expression of myeloid antigens, was insensitive to 2.0% DMSO, in which growth was also limited.

The use of the variant lines in studies of myelopoiesis is as follows. The HL60m2 and m4 appear to be intrinsically restricted in their ability to express the AGF4.36 and 4.48 antigens and can be used to investigate the regulation of expression per se of these antigens at the cell surface. Furthermore, these lines concomitantly show a reduced ability to mature. The line may reflect the interdependence of the controls which regulate the process of neutrophil maturation and the expression of myeloid antigens. Hence the expression of surface antigens can be studied in relation to the regulation of cell maturation. The Ast 1 line appears to be unable to differentiate into neutrophils and may provide insight into whether the presence of a certain surface component is essential for the development of a particular differentiated line. Finally, if physiological inducers of neutrophil maturation were available the lines can be used to investigate the role of the AGF4.36 antigen.

In conclusion, the combined use of cloned populations of cells and markers for the proliferative ability and maturation of cells may be used to investigate the regulation of myelopoiesis. A criticism is that in working with permanent cell lines the events may not reflect normal differentiation. However, the loss of the transferrin receptor as the HL60 cells stop proliferating and the AGF4.36 antigen as the cells mature parallels normal myeloid maturation. Insight, from cell line studies, to the intracellular regulation of these changes will be pertinent to normal myelopoiesis.

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Mechanisms for Induction of Differentiation in the Human Promyelocytic Cell Line HL-60

I. L. Olsson, T. R. Breitman, M. G. Sarngadharan, and R. C. Gallo

Acute myeloid leukemia (AML) is characterized by a block in cell differentiation. Leukemic cell lines which grow continuously in vitro can be used to explore regulation of differentiation in leukemia. The promyelocytic HL-60 line [3] is induced to mature into granulocytes by incubation with agents such as dimethylsulfoxide (DMSO) [4] and retinoic acid (RA) [2]. The mechanisms by which these and other agents induce differentiation are unknown. Knowledge of them could improve understanding of the defects in terminal differentiation characteristic of AML. We have concentrated on mechanisms of action of RA, DIF (see below), and cAMP-inducing agents [1] because these act at low concentrations and most of them are regarded as physiological. Nitroblue tetrazolium (NBT) reduction has been used as the parameter of induced differentiation as it is a reliable measure of functional maturation in HL-60.

Mitogen-stimulated human mononuclear blood cells release polypeptide factors called differentiation-inducing factors (DIFs), which induce HL-60 cells to mature into phagocytizing cells with the morphological characteristics of granulopoietic or myelomonocytic cells [5]. The T-lymphocyte line HUT-102 is a reliable constitutive producer of DIF [8]. HUT-102 supernatant was used for partial purification of DIF employing chromatography on DEAE-Sepharose, blue Sepharose, and Sephadex G-75 followed by electrophoresis in polyacrylamide gels. The HUT-102 produced DIF is a polypeptide with an apparent molecular weight of 58,000. An activity which cochromatographs with DIF acts synergistically with RA to induce maturation not only of HL-60 but also of the monoblast-like cell line U-937 (measured as percentage of cells reducing NBT). Thus, the combination of 10 n*M* RA (which alone gives 20% maturtion of HL-60) and DIF (at a concentration which alone is inactive) induced the maturation of 70%–80% of HL- 60 cells. The combination of 100 n*M* RA (which alone give 5% maturation of U-937) and DIF (which alone is inactive) induced the maturation of 50% of U-937 cells. The synergistic effect between RA and DIF indicates that these agents act by different mechanisms to induce differentiation.

The effect of RA on both HL-60 [1] and U-937 [6] is potentiated not only by DIF but also by agents which increase the intracellular level of cAMP such as prostaglandin E (PGE) and choleratoxin. We found that cells can be primed for differentiation by pretreatment for approximately 1 day with RA followed by exposure to a cAMP-inducing agent or DIF [6]. The reverse sequence was ineffective. Thus, HL-60 could be primed by incubation for 15–20 h with 10 n*M* RA to respond by maturation to the addition of 10 m*M* PGE_2 or 1 n*M* choleratoxin while 10 n*M* RA alone was almost inactive [7]. RA-primed HL-60 also responded to DIF, which alone was inactive at the concentration used in these experiments. U-937 primed by incubation for 24 h with 100 n*M* RA responded to cAMP-inducing agents and DIF, which alone were inactive on this cell line. A decrease in synthesis of some protein(s) seems to favor RA-induced differentiation because priming with RA occurs even better

at a concentration of cycloheximide that inhibits growth completely. However, the continuous presence of the latter agent inhibited maturation. Another finding was that HL-60, but not U-937, primed with RA responded with maturation to ATP and other nucleoside triphosphates, suggesting a role of phosphorylation reactions at the plasma membrane.

Thus our results indicated that cAMP-inducing agents are potent modulators of RA-induced differentiation of both HL-60 and U-937. Therefore a cAMP-dependent phosphorylation reaction(s) might modulate the differentiation response to RA. RA could induce a cAMP-dependent protein kinase (PK) or a substrate which is phosphorylated in the process of differentiation. Treatment of HL-60 for 8–16 h with RA gave a dose-dependent increase in cytosol cAMP-dependent PK I while PK II was decreased. Typical functional changes occur in the plasma membrane as a result of terminal maturation, leading to a capacity for motility, phagocytosis, and secretion. An increase was found in cAMP-independent PK activity of a fraction enriched in plasma membranes from HL-60 treated for less than 1 day with RA. Concomitantly a change in the phosphoprotein pattern of the plasma membrane was observed judging from SDS electrophoresis after incubation of membranes with γ-^{32}P ATP. These changes were seen before morphological maturation. Therefore they may be related to the mechanisms for induction of differentiation.

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Differentiation of a Human Myeloid Cell Line (HL-60) Toward Granulocyte- and Macrophage-like Cells: Comparison of Cell Surface Antigen Expression

B. Uchańska-Ziegler, P. Wernet, and A. Ziegler

A. Introduction

Human hematopoietic pathways have been defined mainly by the analysis of cellular morphological changes because of the paucity of other meaningful markers. Monoclonal antibodies against human cell surface antigens offer the possibility of following changes in the expression of these molecules, in particular when combined with the use of cell lines able to differentiate in vitro if provided with an appropriate stimulus. The aim of this work was to correlate antigenic changes on the surface of differentiating myelomonocytoid cells with the disappearance or appearance of morphologically distinct cell types during hematopoietic differentiation. The promyelocytic leukemia-derived cell line HL-60 was employed as an effective model system. This cell line [4] can be induced with retinoic acid to differentiate toward granulocytes [3], and after the addition of the phorbol ester TPA to HL-60 cells, they become macrophage-like [8]. The expression of surface antigens on these cells following the different types of induction treatment was analyzed with a panel of over 70 monoclonal antibodies [12], using indirect immunofluorescence and bacterial binding assays.

B. Materials and Methods

HL-60 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, antibiotics, and glutamine. They were induced with 1 μM retinoic acid, and the antigenic changes accompanying differentiation toward mature granulocyte-like cells were analyzed for 6 days by indirect immunofluorescence techniques [11] employing a panel of monoclonal antibiodies, of which representative examples are show in Table 1. Simultaneously the morphology of cells was determined with cytocentrifuge preparations.

Induction of HL-60 cells toward macrophage-like cells was performed with 160 nM TPA, and the differentiating cells were characterized for 3–4 days by indirect bacterial binding assays [12].

C. Results and Discussion

The approach outlined here has made it possible to identify a number of antigenic determinants which are expressed to different degrees on the cell surface of myeloid and monocytoid cells, depending on the state of cellular differentiation. The results from the different types of induction experiments show that surface antigens on differentiating HL-60 cells can be assigned to at least seven categories (Table 2). (1) Antigens are not detectable on any of these cells (e.g., certain B-cell antigens like TÜ1). (2) Antigens are present on all cells irrespective of differentiation pathway and extent of differentiation (e.g., tissue common antigens and HLA-A, C heavy chains). (3) Both types of induction lead to the loss of antigens from the cell surface (e.g., the TÜ12 and TÜ15 antigens). (4) Only certain cell types appearing after inductions bear the antigenic determinants (e.g., M1/70.HL antigen). (5) Induction toward granulocytes leads to a higher percentage of antigen-

Table 1. Monoclonal antibodies and their specificity

Monoclonal antibody	Antigen or cell type detected	References
W6/32.HL	HLA-A, B, C, heavy chains	[2]
W6/32.HK	Inactive variant	[13]
TÜ48	HLA-Aw23, -Aw24, -Aw32, -Bw4	[5]
TÜ1	B-cell subpopulation, dendritic reticulum cells	[14]
TÜ3	Myeloid cells } different antigens	[10]
TÜ6, TÜ9	Myeloid cells } different antigens	[9]
OKT4	T-helper/inducer cells	[7]
TÜ12	T-cell subset, immature myeloid cells	[10]
TÜ15	Macrophages, immature myeloid, some T, B cells	[11]
M1/70.HL	Mac-1	[1]
TÜ28	Leukocyte subset	[10]
TÜ42	Leukocyte subset, different from TÜ28	[10]

Table 2. Expression of cell surface antigens on differentiating HL-60 cells

Monoclonal antibody or antigen detected	Induction treatment	
	RA[a]	TPA[b]
W6/32.HL	→[c]	→
TÜ48	→	↓
TÜ6, TÜ9, and other myeloid antigens	→	↓
OKT4	→	↓
TÜ12, TÜ15	↓	↓
M1/70.HL	↑	↑
TÜ3	↑	↓
TÜ28	⌒	→
TÜ42	↓ later ↑	↓

[a] RA, retinoic acid; expression determined by indirect immunofluorescence test

[b] TPA, 12-0-tetradecanoyl-phorbol-13-acetate; expression determined by bacterial binding assay

[c] "→" antigenic determinant expressed on an approximately equal percentage of cells during differentiation; "↓" antigenic determinant lost during differentiation; "↑" antigenic determinant appears on a larger fraction of cells during differentiation; "⌒" antigenic determinant expressed during differentiation only on certain cell types; further differentiation causes the loss of this determinant

bearing cells while differentiation towards macrophages causes the loss of the molecule from the cells (TÜ3) antigen). (6) Antigens present on certain cell types of uninduced and retinoic acid induced HL-60 cultures disappear from the cell surface after exposure to TPA (e.g., the myeloid TÜ6 or TÜ9 antigens, HLA-B molecules as detected by TÜ48, as well as the the antigen recognized by OKT4). (7) Antigens show an expression different from those described above (e.g., the TÜ28 or TÜ42 antigens).

It was a surprise to find that HL-60 cells reacted with OKT4, which has been described to be specific for T-helper cells [7], but several other antigens predominantly directed against T cells, like TÜ12 for example, also exhibit activity toward HL-60 cells. The loss of reactivity of TPA-induced cells with TÜ48 simultaneous with retention of HLA heavy chains as detected by W6/32.HL suggests that HLA-B antigens are under separate genetic control. This interesting phenomenon deserves further analysis (see also Ziegler et al., this volume). Table 2 shows that both differentiation pathways lead to the expression of a defined, easily distinguishable set of antigenic determinants, as defined by the panel of monoclonal antibodies employed here. A correlation of antigenic changes with morphologically distinct cell types reveals,

after both types of induction, that antigenic determinants which are lost after induction appear to be absent from more mature cell types while the reverse seems to be true for those antigens which are not expressed on uninduced HL-60 cells. Furthermore, most alterations in the expression of membrane antigens precede morphological maturation. Preliminary experiments with human bone marrow cells indicate that normal cell types exhibit changes in surface antigen phenotype and morphology comparable to those described here for an in vitro model system. Space does not allow a comparison of the results presented here with those of other investigators, but, e.g., Perussia et al. [6] have also used a panel of antibodies for similar purposes.

Further studies with HL-60 cells should be designed to answer questions regarding the molecular mechanisms involved in the regulation of differentiation-related gene expression.

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Transmission of Human T-Cell Leukemia Virus (HTLV) into Human Cord Blood T Cells

P. S. Sarin, M. Popovic, S. Z. Salahuddin, E. Richardson, G. Lange Wantzin, B. Karmarsky, and R. C. Gallo

The isolation of HTLV, a type C retrovirus, was first reported from our laboratory from adult patients with T-cell malignancies [4, 10, 11]. HTLV has now been isolated from a number of T-cell leukemia/lymphoma patients from various parts of the world, including the United States, Caribbean, Israel, and Japan [14]. An identical or extremely closely related type C retrovirus was subsequently isolated by Japanese workers from patients with adult T-cell leukemia (ATL) but has been called by another name, ATLV [5, 9, 18]. Seroepidemiological studies indicate that antibodies to the internal antigens (p19 and p24) of this virus are present in a large number of T-cell leukemia/lymphoma patients and some healthy normal blood donors resident in the endemic and nonendemic areas [1, 2, 5, 6, 17]. Nucleic acid hybridization studies and high incidences of association of this virus with T-cell leukemia indicate that this virus is acquired by exogenous infection ([3]; Gallo et atl., this volume; [15, 18]). HTLV and ATLV have been shown to be identical or extremely closely related by competition radioimmunoassays and radioimmunoprecipitation of the internal antigens (p19, p24) and by nucleic acid hybridization studies [13]. HTLV is to date unique to forms of the adult T-cell leukemia/lymphoma (Gallo et al., this volume). Elsewhere in this book (Gallo et al.) we describe the transmission of HTLV into human cord blood T cells and we show the HTLV-induced changes in cell growth and surface phenotype. We also show evidence for changes in expression of certain genes. Here we show the morphological changes in HTLV-infected T cells, and we report on the decreased requirement for T-cell growth factor (TCGF) after transmission of the virus. The features of the HTLV-infected and transformed cord blood human T cells are remarkably similar to the primary tumor cells of HTLV-associated T-cell malignancies.

A. Transmission of HTLV into Human Cord Blood Cells and Characteristics of the Infected Cells

HTLV was transmitted into the cord blood T cells from the HTLV-positive cell lines by cocultivation of the HTLV-positive cell lines with fresh human cord blood T cells or in a few instances by addition of cell-free virus particles. Briefly, the cord blood leukocyts were purified on Ficol/Hypaque, washed three times with RPMI-1640 containing 10% fetal calf serum, and mixed with HTLV-positive cell lines (MJ, UK, TK, etc.) that have been either exposed to X-rays (6000 rads) or to mitomycin-C (100 μg/ml for 20 min at 37°) and washed three times with RPMI-1640 containing 10% fetal calf serum. The cord blood cells and the X-irradiated HTLV-positive cells were mixed at a ratio of 4 : 1 and incubated at 37 °C in the presence of 5% CO_2 in the presence or absence of 5% TCGF. After 3 weeks, 5 weeks, and 7 weeks of coculture, the cells and the conditioned medium were tested for the expression of HTLV-related proteins (p19, p24) and reverse transcriptase. The cells were also examined by electron microscopy for detailed morphologic characteristics and for the expression of type C virus.

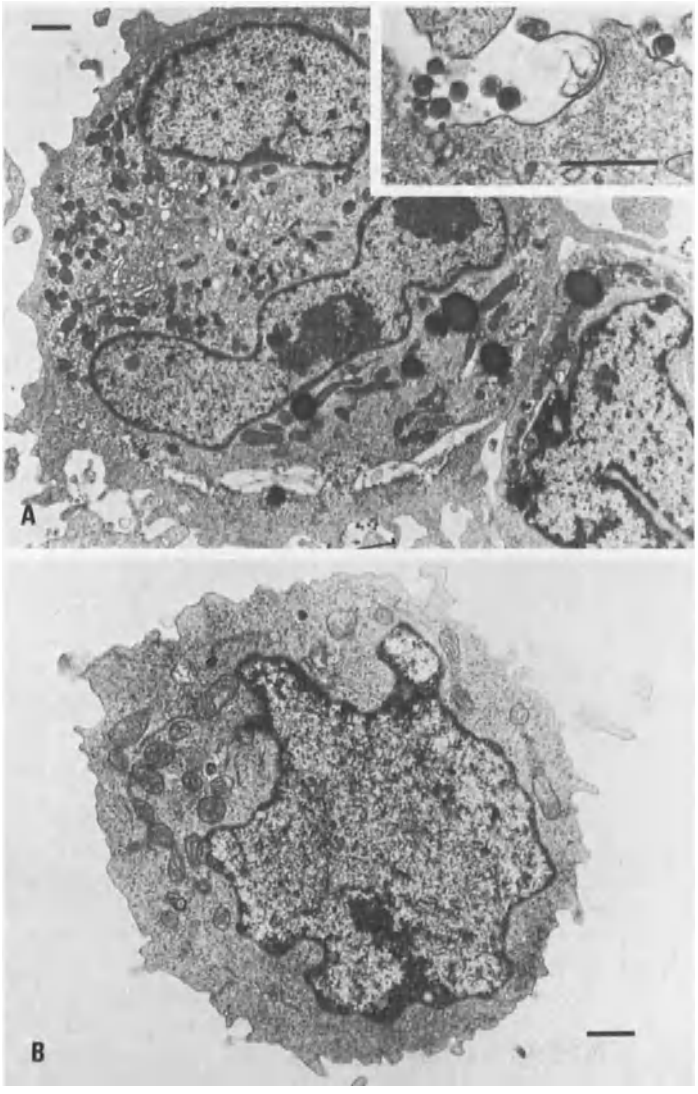
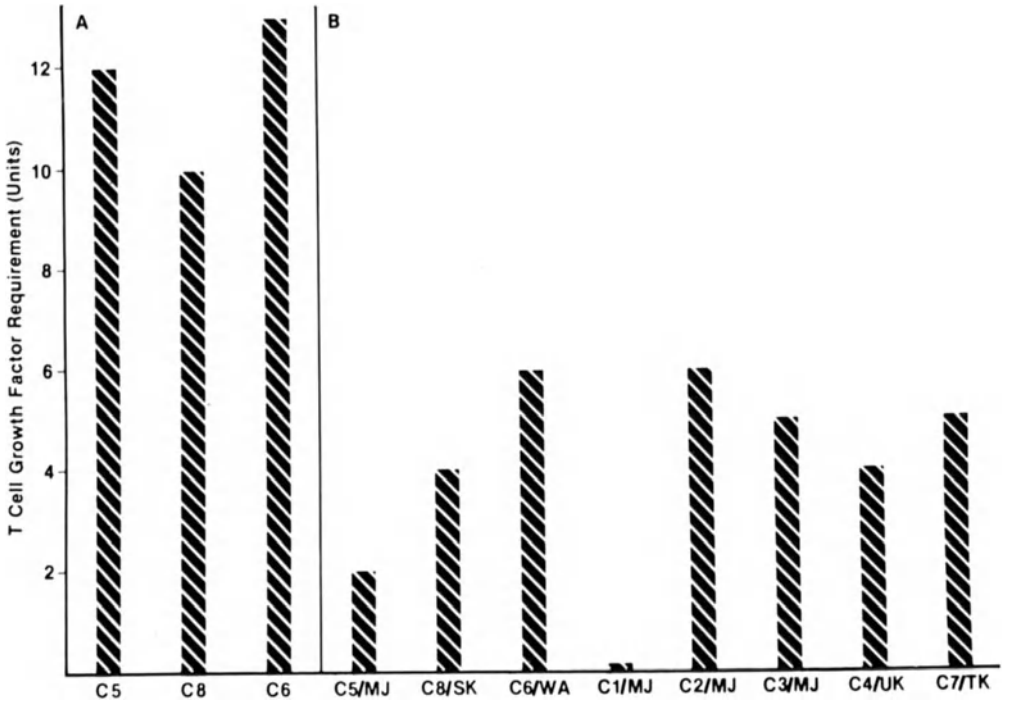


Fig. 1 A, B. Electron microscopic examination of human cord blood T cells before and after infection with HTLV. **A** HTLV-infected cord blood T cells (*insert*). Typical type C virus particles. **B** Uninfected cord blood cells

Nine HTLV-positive cell lines were successfully transmitted into cord blood T cells as observed by the expression of p24, p19, and reverse transcriptase. A representative example of the expression of HTLV into cord blood T cells as seen by electron microscopy is shown in Fig. 1. After infection with HTLV many cord blood T cells develop lobulated nuclei (Fig. 1 A) similar to the morphology of the nuclei in many HTLV-associated primary malignant cells. The *insert* in Fig. 1 A shows the presence of type C virus particles associated with infected cells. A typical normal cord blood T cell is shown in Fig. 1 B. The infected cord blood cells grow as multinucleated giant cells (Fig. 2 C). The presence of multinucleated cells is a common feature of the HTLV-infected cord blood cells. The donor HTLV-positive cell lines also contain multinucleated cells but the size of the HTLV-infected cord blood cells is generally larger and in some cases up to 30 nuclei have been seen in a giant cell.



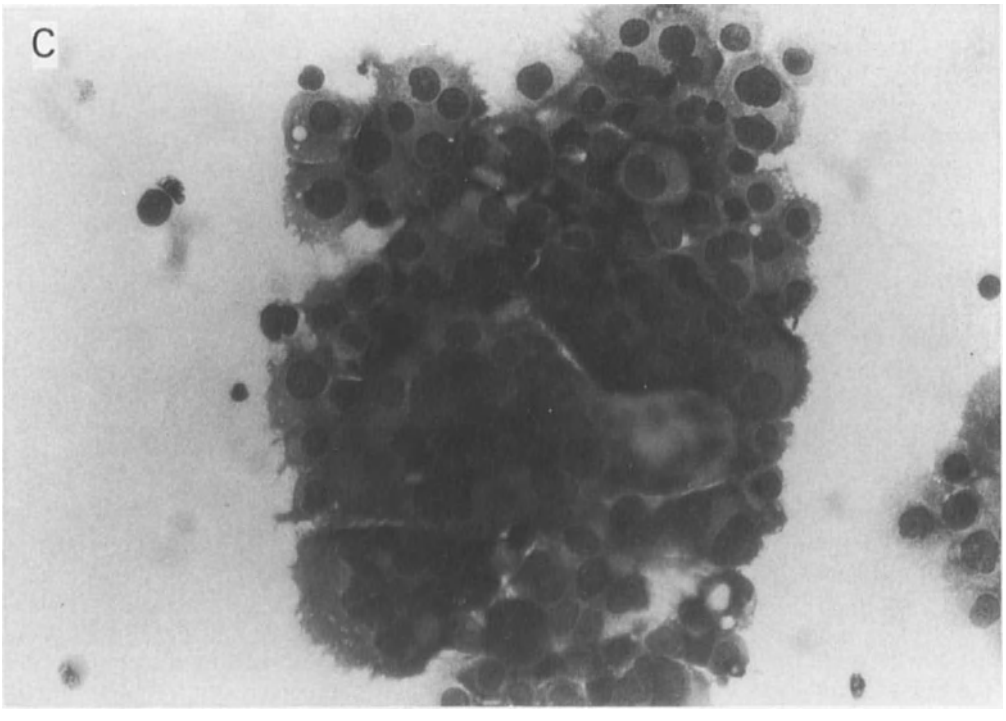


Fig. 2A–C. T-cell growth factor (TCGF) requirement of the uninfected and HTLV-infected cord blood T cells and morphology of the infected cells. **A** TCGF requirement of uninfected cord blood cells. A unit of TCGF is defined as the half maximal incorporation of ^{3}H-TdR in a TCGF microassay. **B** TCGF requirement of HTLV-infected cord blood T cells. **C** Presence of multinucleated giant cells in HTLV-infected cord blood T cells

Table 1. Comparison of the characteristics of HTLV-positive human neoplastic T cells with normal uninfected and HTLV-infected human cord blood T cells

Property	HTLV positive Neoplastic T cell lines	Cord blood T cells	
		HTLV infected	Lectin stimulated
1. In vitro growth	> 180 days	> 180 days	< 50 days
2. Requirement for exogenous TCGF (v/v)	0% – 5%	0% – 5%	10% – 12%
3. TCGF receptors (TAC)[a]	+ + +	+ + +	+
4. E-rosette	+	+	+
5. S-IgG, EBNA, TdT[b]	–	–	–
6. Cell phenotype			
(a) Inducer/helper (OKT4, Leu 3)	10/10	9/9	NT[c]
(b) Suppressor/cytotoxic (OKT8, Leu 2A)	2/10	2/9	NT[c]
7. HTLV p19, p24, and RT[d] expression	+	+	–
8. Type C virus particles (EM)	+	+	–
9. Cell morphology			
(a) Presence of multinucleated giant cells	+	+	–
(b) Presence of lobulated nuclei	+	+	–

[a] TCGF receptors determined by cell sorter with TAC antibody [8, 19]
[b] TdT, terminal deoxynucleotidyl transferase
[c] NT, not tested
[d] RT, reverse transcriptase

B. The TCGF Requirement for Growth

A characteristic feature of the HTLV-infected cord blood cells is the decreased requirement for TCGF for growth [4]. As shown in Fig. 2 A and 2 B the normal cord blood T cells require 10%–12% (v/v) TCGF for growth whereas the HTLV-infected cord blood T cells can grow in 0%–5% (v/v) TCGF. In one case C_1/MJ (cord blood cells infected with HTLV-positive T-cell line MJ) less than 1% of TCGF is required for growth. More recently HTLV has also been transmitted into adult T cells by using conditions similar to those described above. Some of the HTLV-infected T cells have become TCGF independent and are expressing HTLV proteins (p19, p24), reverse transcriptase, and type C virtus particles. Further evaluation of these cell lines is currently in progress.

C. Characteristics of Normal and HTLV-Producing T-Cell Lines

The characteristic features of HTLV-positive primary cell lines obtained from patients with T-cell leukemia and the cord blood T cells before and after infection are summarized in Table 1. The normal cord blood T cells reach a crisis period at 45–50 days (Gallo et al., this volume) whereas the HTLV-positive T-cell lines and HTLV-infected cord blood T cells grow for indefinite periods. The normal cord blood cells require more TCGF for growth (Fig. 2) and have a lower number of TCGF receptors than the HTLV-positive primarly T cell lines and HTLV-infected cord blood T cells (Table 1).

The current studies show that HTLV-positive T-cell lines and HTLV-infected cord blood cells possess mature T-cell markers (OKT4 positive, E-rosette positive, terminal transferase negative), grow as multinucleated giant cells, contain lobulated nuclei, need less TCGF for growth compared with normal T cells, and express HTLV antigens (p19, p24) and HTLV particles. All these features are remarkably similar to the characteristics of primary tumor cells from HTLV-positive T-cell leukemia patients. This system may offer the possibility of investigating the cellular and molecular pathogenesis of a human malignancy in vitro in a manner not previously available for a human cancer.

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Modern Trends in Human Leukemia V
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K 562 Cell Line in Plasma Clot Diffusion Chambers: Changes in Cell Surface Phenotype in Relationship to Culture Conditions

B. Lau, G. Jäger, E. Korge, and P. Dörmer

A. Introduction

It is well known that proliferation and subsequent differentiation of normal hemopoietic progenitor cells require an appropriate inductive microenvironment and the addition of specific regulatory factors [7]. This is also true for differentiation of leukemic blast cells, which are considered to represent early stages of a hemopoietic cell lineage [6].

In order to examine whether the phenotypic characteristics of the K 562 cell line originally established from cells during blast crisis of a chronic myelocytic leukemia [4] can be influenced by environmental conditions of the culture, we ued the in vivo plasma clot diffusion chamber (DC) technique [8]. Normal CBA mice (group A) as well as animals rendered anemic with phenylhydrazine (group B) served as DC recipients.

B. Material and Methods

The K 562 cell line, kindly provided by Dr. L. C. Andersson, Helsinki, was maintained in RPMI 1640 medium plus 15% heat-inactivated fetal calf serum and antibiotics. Cells grew in a roller suspension and were adjusted to $1-1.5 \times 10^6$ cells/ml.

According to the method of Steinberg et al. [8] DCs (pore size 0.22 μ) were filled with 5×10^5 K 562 line cells suspended in 100 μl TC medium 199. Thereafter, 20 μl of citrated bovine plasma was added to each chamber, two of which were inserted into the peritoneal cavity of each CBA mouse (25–30 g). Animals in group A were injected intraperitoneally with 0.1 ml isotonic NaCl solution either 24 or 3 h prior to the implantation of chambers. The same time schedule was applied to CBA mice in group B, which had been pretreated with phenylhydrazine (50 mg i.p./100 g body wt.). Hematocrits and reticulocytes were determined from free-flowing tail blood of the DC recipients.

At different instances during the culture period DCs were removed, and were shaken in a 2.0% pronase solution. Besides total and differential counts one part of the chamber yield was processed for cell surface characterization with heterologous rabbit anti-glycophorin A antiserum [1], and rabbit anti-human granulocyte antiserum [3]. Evaluation was performed by applying indirect immunofluorescence. To avoid unspecific binding, all cells were preincubated with aggregated human IgG. Additionally, in situ hybridization of globin mRNA was performed using ^{3}H-cDNA derived from rat globin mRNA. Grains over individual cells were counted by incident light microphotometry [2].

C. Results and Conclusions

Whereas the hematocrits and reticulocytes of mice in group A ranged within normal levels, these parameters changed considerably in animals of group B which had been pretreated with phenylhydrazine. In this group, hematocrit values dropped from an average of 47% to a mean of 27% between day 1 and day 3 of the culture period. At the same time the number of reticulocytes increased up to 95%.

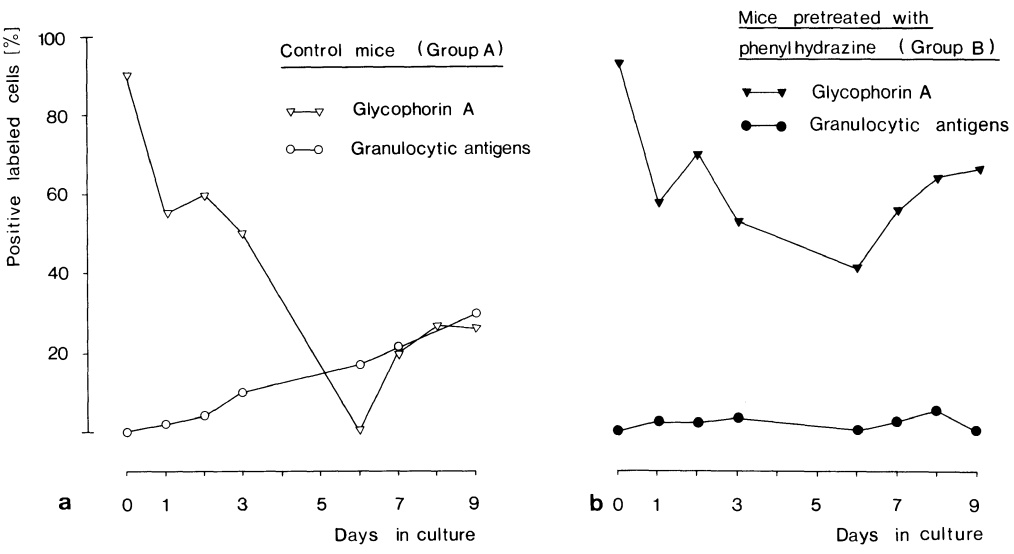

Fig. 1. Cellular phenotype characteristics of K 562 cells during a 9-day plasma clot DC culture in normal **a** and anemic **b** mice

In spite of these hematological perturbations, the growth pattern of K 562 cells was quite similar in both groups. It could be characterized by an increase in cell number until day 6, after which the chamber content obtained from anemic mice decreased slightly more than that from animals in group A. Morphological examination revealed no changes in the blast-like appearance of K 562 line cells under both culture conditions. However, differences were observed in the immunological characteristics of the chamber yield in groups A and B. As illustrated in Fig. 1, a high percentage of the chamber input positively labeled with the anti-glycophorin A antibody. After a gradual decline of glycophorin A positive cells during culture in anemic animals, there was a continuous increase from day 6 onward, reaching 70% at the end of the observation period. By using normal mice as DC hosts in group A, cells carrying glycophorin A completely disappered on day 6. Thereafter, these cells reappeared but did not exceed 30% toward the end of the culturing experiment. Concerning the reaction pattern with anti-granulocyte antiserum, a clear difference in the percentage of positive cells occurred from day 6 on, depending on whether normal or anemic mice served as DC recipients. In group A mice (Fig. 1 a) the number of cells characterized by the presence of granulocytic antigens increased up to approximately 30% on day 9, whereas few or no positive cells could be seen during culture in mice of group B (Fig. 1 b).

In accordance with the immunological findings, an increased content of globin mRNA was detected by in situ hybridization of K 562 line cells from group B. The maximum of grain counts which were equally well distributed over the nuclei and the cytoplasm was seen between day 6 and 9 of the culture.

Our observations provide evidence for a modulation in the differentiation of K 562 line cells depending on environmental conditions during plasma clot DC culture. The expression of granulocytic antigens, on one hand, and the maturation along the erythropoietic lineage, on the other hand, support the hypothesis of a bipotential differentiation capacity of K 562 line cells, a hypothesis which is in line with the findings of other groups [5].

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Antigen Expression on Normal and Leukaemic Erythroid Precursors

C. Sieff, D. Bicknell, G. Caine, P. A. W. Edwards, and M. Greaves

A. Introduction

Erythroleukaemia is rare, comprising no more than 5% of acute myeloid leukaemias. This low incidence is curious, since the involvement of different cell lineages in leukaemia is likely to reflect progenitor cells "at risk" of transformation, and myeloid progenitors do not occur with a greater frequency than those of the erythroid lineage. One possible explanation is that myeloid progenitors are more "sensitive" target cells for leukaemogenesis than erythroid progenitors. However, clonal analysis has shown that although acute myeloid leukaemia can involve a target cell with differentiation restricted to the granulocyte macrophage lineage, it may frequently involve a pluripotent stem cell target [5, 11, 20], and therefore the limited maturation observed is preferentially non-erythroid.

An alternative explanation, suggested by Andersson [1], is that erythroleukaemia is in fact more common than we realise, but that it is incorrectly diagnosed as poorly differentiated acute myeloid (M1-FAB classification) [4] or even acute lymphoblastic leukaemia. Using a rabbit antiserum specific for the majored cell membrane protein glycophorin A, he found that 15% of M1 AML and 10% of relapsed ALL were glycophorin A positive. Some cases were also positive with an antibody to fetal haemoglobin.

We have used a series of monoclonal antibodies, including two specific for glycophorin A, together with fluorescence-activated cell sorting and clonal cultures of haemopoietic progenitors to determine the pattern of antigen expression during normal erythropoiesis, and have compared this with the pattern observed in acute leukaemias.

B. Methods

Normal bone marrow was obtained by aspiration from adult volunteers, centrifuged on Ficoll-isopaque, and the interface mononuclear cells collected, washed and suspended in Eagles medium +2% fetal calf serum. $20–100 \times 10^6$ bone marrow cells were incubated for 30 min at 4 °C with previously determined optimal concentrations of monoclonal antibody (see below), washed and then stained with an affinity purified $F(ab')_2$ preparation of goat anti-mouse antibodies which had been cross absorbed with insolubilized human immunoglobulin and labelled with fluorescein isothiocyanate (FITC). The cells were washed twice and then analysed and sorted on a modified fluorescence activated cell sorter (FACS-1, Becton Dickinson). Cells were processed in sterile conditions using relative fluorescence intensity to separate positive from negative cells. Cytospin preparations were made from aliquots of the unfractionated control and from each fraction, stained with May-Grünwald-Giemsa, and differential leucocyte counts performed.

Culture Procedures. Unfractionated controls and positive and negative fractions were cultured for erythroid colonies in methyl cellulose [12] in the presence of 2–2.5 u erythropoietin (Connaught Step III); erythroid colony forming units (CFU-

E) were counted at 7 days and burst-forming units (BFU-E) at 14 days.

Granulocyte-macrophage colonies (CFU-GM) were cultured in a mixture containing 25% FCS, 1% BSA, antibiotics, 0.9% methylcellulose and 5% PHA-leucocyte conditioned medium and CFU-GM counted on day 13 or 14. Recovery per 10^5 unfractionated cells was determined for each fraction and expressed as a percentage of the total colony recovery.

A similar analysis was carried out for precursors using the cytospin differential counts.

Leukaemic samples (heparinized blood and bone marrow) were obtained from referring hospitals throughout the United Kingdom as part of a routine immunodiagnostic service. All samples were separated on Ficoll-isopaque and binding of murine monoclonal antibodies assessed with FITC $F(ab')_2$ goat anti-mouse IgG using both fluorescence microscopy (Standard 16 Zeiss photomicroscope with epi-illuminescence) and flow cytometry (FACS-I).

For intranuclear TdT staining cytospin slides were fixed in cold methanol, incubated with rabbit antibodies specific for TdT [6], followed, after washing, by fluorochrome-labelled goat antibodies specific for rabbit IgG. The E rosette test was performed by standard methods using neuraminidase-treated sheep red blood cells.

C. Antibodies

I. Mouse Monoclonal Antibodies

LICR.LON.R.10 and LICR.LON.R.18 were produced as previously described [2, 10]. Ascitic IgG was used and titred by indirect immunofluorescence using erythrocytes, the cell line K562 [13] and the FACS. The dilution used was twice the minimal concentration giving saturated (maximal) staining intensity.

Other monoclonal antibodies used include J-5 anti common-ALL [17]; OKT1, OKT3, OKT6, OKT9, OKT10, and OKT11a [14, 16]; DA-2 anti HLA-DR and W6/32 anti HLA-ABC [8] and AN51 anti-platelet glycoprotein I [15]; OKM1 anti-monocyte/granulocyte [7]; R6A-anti band 3 [2]; and MAS 016 [3] and N16 [9] anti-blood group A.

II. Human Monoclonal Antibodies

Human Monoclonal antibodies to blood group I (Stephenson) and i (Dench) were obtained from Dr. K. Shumach (Toronto General Hospital).

III. Rabbit Anti-Human Transferrin

(DAKO).

D. Changes in Cell Surface Antigen Expression During Haemopoietic Differentiation

The patterns of antigen expression during normal haemopoiesis have been established using antibodies defining blood group (A, I/i), HLA-associated (-ABC and -DR), lineage-specific and transferrin receptor antigens. Details of these results are published elsewhere [18, 19] and summarized in Fig. 1.

Like HLA-DR and ABC the antigen defined by OKT10 is expressed on the earliest progenitors and lost during differentiation, suggesting a possible role in interactions regulating the proliferation of these cells.

In contrast, blood group A antigen is strongly expressed on only a small proportion of early erythroid and myeloid progenitors but increases during differentiation. I antigen shows a similar increase in expression during erythropoiesis but little expression on myeloid cells. Conversely, i antigen shows little expression on erythroid progenitors but is expressed on erythroid precursors and on myeloid progenitors and precursors.

Erythroid lineage specific antigens glycophorin A (gA) and band 3 are not present on erythroid progenitors and first expressed on maturing normoblasts. Similar results for gA expression have now been obtained using the monoclonal antibody R.10 and a rabbit anti-gA kindly provided by Dr. L. Andersson. There is no significant expression of these determinants on myeloid cells.

Transferrin receptors, defined by an antibody to transferrin itself or by the monoclonal OKT9, are expressed on most BFU-E and virtually all CFU-E but only a smal-

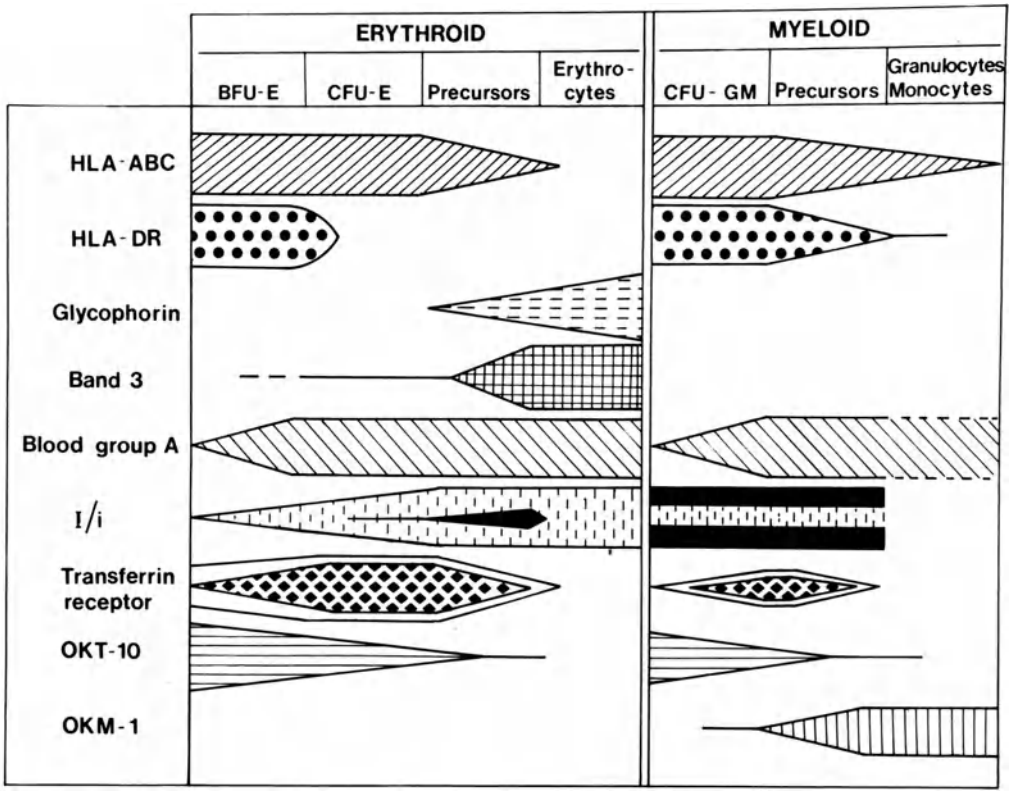

Fig. 1. Summary of monoclonal antibody defined cell surface phenotype of erythroid and myeloid cells. *Width of bars* approximates to relative proportion of antigen-positive cells in various maturation compartments. Blood group I/i: *solid, black area* represents i expression. Transferrin receptor: *hatched area* represented expression of monoclonal OKT9 defined determinant compared with the transferrin-binding site (*total area*)

ler proportion of myeloid cells and therefore may be useful for selecting and enriching for erythroid progenitors. They are not, however, specific for cells of the erythroid lineage.

E. Monoclonal Anti-glycophorin as a Probe for Erythroleukaemia

Since it is evident from the above data that we do not have an antibody specific for erythroid progenitors, it is clear that for the purpose of comparison with leukaemic phenotypes these data are incomplete; erythroleukaemias originating in either pluripotent stem cells or early erythroid progenitors, and those with maturation 'arrest' at these stages of differentiation, would not be expected to be gA+, unless asynchromy of gene expression had occurred. Furthermore, they would not be diagnosable as erythroid unless they could be induced to differentiate to gA+ or haemoglobin synthesizing cells in vitro. Seven hundred and fifty one cases of leukaemia, either at presentation or relapse, were assessed for glycophorin positivity. A variable proportion of gA+ cells were present in 27 erythroleukaemias studied; six with a high proportion of blasts had less than 15% gA+ cells while 21 had between 16% and 66% positive cells.

The overwhelming majority of non-erythroleukaemias were glycophorin A negative (Table 1), i.e. less than 10% positive cells, and this included 224 patients with acute lymphoblastic leukaemia at presentation and 103 in relapse. Four cases out of 81 common ALL in relapse had a marked increase of gA+ cells (up to 65%), but these patients were in early relapse

Table 1. Reactivity of different leukaemias with monoclonal anti-glycophorin

	Reactive	Unreactive	
		Presentation	Relapse
Erythroleukaemia	27		
Acute lymphoblastic leukaemia	2[a]		
Common ALL		149	81 (4E)[b]
T-ALL		32	10
Null-ALL		41	12
B-ALL		2	0
Acute undifferentiated leukaemia		23	4
Acute myeloid leukaemia	2+5E[b]	152	15
Acute promyelocytic leukaemia		9	0
Acute myelomonocytic/monocytic leukaemia	1	28	2
Acute megakaryoblastic leukaemia	2	3	2
Chronic myeloid leukaemia (CML)		14	0
CML blast crisis			
Erythroid	3		
Lymphoid	0	0	33
Myeloid	4	0	68
Megakaryoblastic	1		
Other		15	9

[a] Both baby girls less than 6 months old with equivocal diagnosis of ALL (see text)

[b] Clearly defined erythroid component separate from blast cells

with only a moderate increase of lymphoblasts and significant residual erythropoiesis. Separation of the gA+ cells in one case using the FACS identified greater than 85% of these cells as normoblasts. Two patients, both girls less than 6 months old, were diagnosed and treated as ALL but had a high proportion of gA+ blasts (Table 2). There was no immunological evidence to support the diagnosis of ALL (Table 2) and review of the morphology and cytochemistry showed pleomorphic blasts with basophilic-vacuolated cytoplasm and prominent granular PAS positivity. Neither patient responded to standard induction treatment for ALL, and more intensive therapy failed in the one patient who received it; both patients died without remitting. It is likely that both of these patients were in fact 'cryptic' erythroleukaemias.

There were only 18 gA+ cases out of 371 non-lymphocytic, non-erythroid leukaemias studied and none of 24 other cases including B-CLL, disseminated lymphomas, myeloma and hairy cell leukaemia (one case). Eight patients of 214 with acute myeloid leukaemia had between 15% and 50% gA+ cells; however, an obvious erythroid component was present in five of these cases. Two patients of seven with acute megakaryoblastic leukaemia were gA+; one of these patients had two cell populations demonstrable by fluorescence mi-

Table 2. Antigenic characteristics of leukemic blasts in two cases of putative 'cryptic' erythroleukemia diagnosed as ALL

Cell markers	Patient	
	D.H.	S.H.
Monoclonal antibodies		
J-5 (anti-cALL/gp100)	–	–
DA-2 (anti-HLA-DR)	–	9%
OKT1, 3, 4, 6, 8, 11A (anti-T)	–	16% – 19%
AN51 (anti-platelet glycoprotein I)	–	n.t.
OKM-1 (anti-granulocyte/monocyte)	–	–
R6A (anti-band III)	7%	n.t.
LICR.LON.R10 (anti-glycophorin A)	85%	81%
R.18 (anti-glycophorin A)	86%	n.t.
Other markers		
Sheep (E) rosettes	–	8.5%
Cell surface Ig	–	7%
TdT	–	–

– = < 5% positive cells
n.t. = not tested

croscopy and FACS analysis, large blast cells positive with the antiplatelet monoclonal AN51 and smaller gA+ blasts. This leukaemia appears to involve a progenitor common for the erythroid and platelet lineages. Eight Ph' positive blast crises of chronic myeloid leukaemia (of 109 tested) were gA+. Three of these were, however, 'erythroid' blasts crises, four myeloid and one possibly megakaryoblastic, but was negative with the platelet glycoprotein I monoclonal AN51. These data therefore differ from those of Andersson et al. [1], the number of gA+ leukaemias being considerably less than indicated in the smaller series from Finland. Part of the explanation may lie in differences in diagnostic criteria. Of the small number of AMLs that had gA+ cells in our series, most had a morphologically identifiable erythroid component, and the two interesting gA+ paediatric cases were considered on haematological grounds to be possible, though not unequivocal ALLs. Immunological data indicated that they were not ALL. Another possible explanation for the discrepancy could be differences in the antibodies used to detect gA, but this is unlikely since we found similar intensities and pattern of staining in comparative studies with rabbit anti-gA (kindly provided by L. Andersson).

We conclude from our data that the highly selective expression of gA in normal haemopoiesis is maintained in leukaemia. Glycophorin-positive leukaemias may be overt erythroleukaemias (M6 AML) or mixed leukaemias with erythroblasts and megakaryoblasts, suggesting clonal origin from a bi- or pluripotent stem cell (megakaryoblastic leukaemias and Ph' blast crisis of CML).

Only very rarely do cases diagnosed as AML or ALL express glycophorin A, and we suggest than these cases are genuine erythroleukaemias and not myeloblasts or lymphoblasts aberrantly expressing gA.

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The Early Stage of Friend Virus Erythroleukemias: Mechanisms Underlying BPA-"independent" In Vitro Growth of BFU-E*

C. Peschle, G. B. Rossi, A. Covelli, G. Migliaccio, A. R. Migliaccio, and G. Mastroberardino

The erythroleukemias induced by Friend virus (FV) are characterized by a stepwise progression toward malignancy. In the *first stage*, i.e., the initial 3 weeks postinfection (p.i.), the mice show a pronounced wave of erythroblastosis in spleen, marrow, and blood [5]. Cell lines could not be established from these animals [12]. In the *second stage* (i.e., after 21 days p.i.) overt malignancy develops, as permanent cell lines can be easily obtained [12]. These are indistinguishable from the original Friend erythroleukemia lines [5].

At least two strains of FV are presently known. The first one (FVA) induces an erythroleukemia with splenomegaly, enhanced but ineffective erythropoiesis, and mild anemia [4]. The second one (FVP) causes the same type of leukemia, associated with effective erythropoiesis and marked polycytemia [13].

An *onc* gene has been demonstrated in "acute- type" RNA tumor viruses [7], but not so far in the FV complex. Indeed, the genomes of FVP and FVA consist of two components: a replication-defective spleen focus-forming virus ($SFFV_P$ and $SFFV_A$, respectively) and a replication-competent murine leukemia virus ($F\text{-}MuLV_P$ and $F\text{-}MuLV_A$, respectively) [20]. The two components have been recently cloned [9, 14, 15]. Injection of $F\text{-}MuLV_P$ or $F\text{-}MuLV_A$ into newborn Balb/c or NIH/swiss mice induces an erythroleukemia with splenomegaly and anemia [11, 14, 15]. Treatment with cloned $SFFV_P$ or its *"env"* fragment with LTR, in association with any of different helper viruses, induces in newborn and adult susceptible animals an erythroleukemia with splenomegaly, erythroblastosis, and polycythemia [10]. In contrast, $SFFV_A$ has little or no biological activity, perhaps due to defective glycosilation of its gp52 marker [19].

Our studies have been focused on the kinetics of early (BFU-E of *primitive* type) and late (CFU-E) erythroid progenitors in the *first* stage after FVP and FVA infection. In this regard, the kinetics of normal hemopoietic progenitors is controlled by specific hemopoietins. In vitro cycling and differentiation of BFU-E is largely modulated by burst-promoting activity (BPA), i.e., a glycoprotein factor of ~24,000 daltons [8, 21, 22]. CFU-E kinetics is largely regulated by erythropoietin (Ep) [6]. Proliferation of granulomacrophage progenitors (CFU-GM) is modulated by colony-stimulating factors (CSF) [2].

The first stage of FV erythroleukemia is characterized by marked amplification of the splenic pool of BFU-E [18] and CFU-E [16]. The cycling activity of the former progenitors is markedly enhanced [18]. In vitro growth of CFU-E from mice treated with FVP does not require Ep addition [3, 16, 18].

The enhanced cycling and perturbed kinetics of BFU-E in FV mice are compatible with a rise of BPA. Results obtained in our laboratory indicate that this elevation occurs. Indeed, in vitro growth of spleen BFU-E from 1-, 2-, and 3-week infected animals is partially or totally independent

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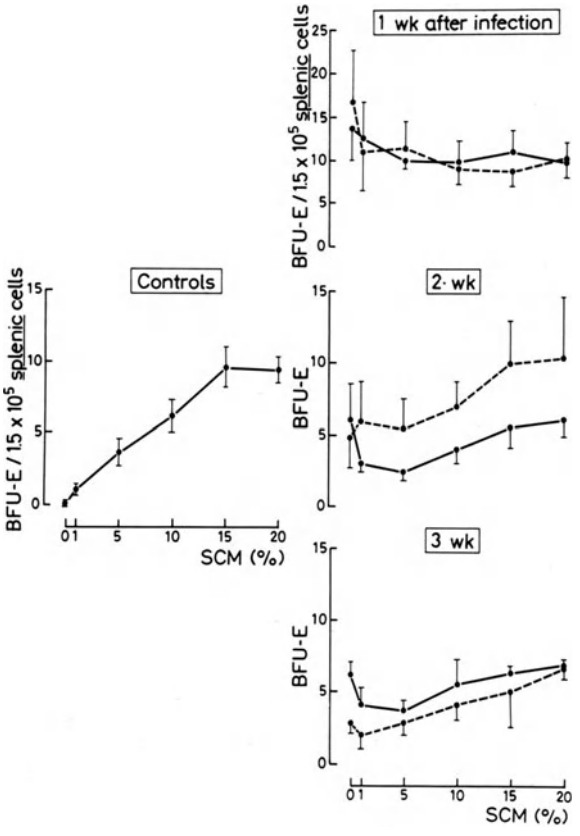

Fig. 1. SCM (see text) dose/response curve in 4% fetal calf serum cultures [8] of BFU-E from spleen of normal (controls) and 1-, 2-, 3-week infected FVP (——) or FVA (– – –) mice. Mean ± SEM values are presented (three to six experiments/points, two plates/group in each experiment)

of exogenous BPA (i.e., lectin-stimulated spleen-conditioned medium, SCM) (Fig. 1). This hormone "independence" is less clearly expressed at marrow level (Fig. 2 and data not shown). In contrast, cloning of CFU-GM from infected mice strictly requires CSF addition ([17], and results not presented here).

The "BPA independence" of BFU-E is apparently due to an in vitro rise of BPA, in turn mediated via two synergistic mechanisms: (1) hypersensitivity of BFU-E to BPA, as suggested by SCM dose-response curves for marrow BFU-E (Fig. 2). (2) Increased BPA release in culture. This is suggested by nonlinearity of cell/colony regression of BFU-E in absence of SCM (Peschle et al., in preparation), and increased BPA in medium conditioned by splenocytes from FVP-infected animals as compared to appropriate controls (Peschle et al., in preparation).

Growth of CFU-E from FVP-treated mice in serum-free cultures is largely independent of exogenous Ep [17], as previously reported. Indeed, these progenitors show marked hypersensitivity to Ep, up to independence of it (Peschle et al., in preparation). It is tentatively postulated that the rise of BPA, particularly in spleen, may play a key pathogenetic role in the early stage of FV erythroleukemias. Indeed, FV causes both (a) a rise of BPA and (b) erythroblastosis progressing into erythroleukemia. These two phenomena may either develop in parallel, or be linked by a cause/effect relationship. In the latter hypothesis the following aspects are important. Elevated BPA induces enhanced cycling of BFU-E [22]. Additionally, a glycoprotein possibly identical to BPA triggers proliferation of CFU-S [22]. The cycling BFU-E is considered a suitable target for infection and transformation by FV [1]. On this basis, the following sequence of events may hypothetically underlie FV erythroleukemias: (1) FV enhances production of BPA, particulary in the spleen. (2) Ele-

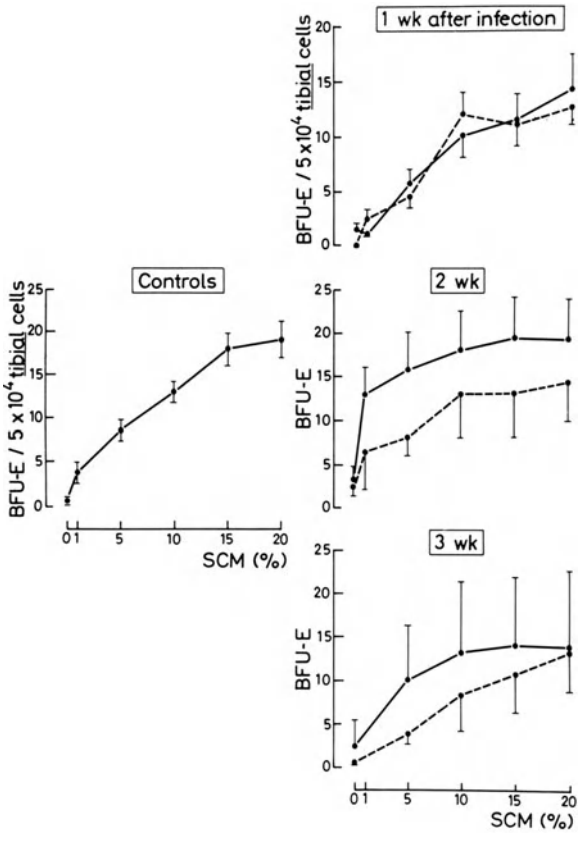

Fig. 2. SCM dose/response curve of tibial BFU-E (for details see Fig. 1): BPA hypersensitivity of BFU-E from 2- and 3-week infected FVP animals and possibly 1- and 2-week infected FVA mice

vated BPA forces quiescent BFU-E (and possibly CFU-S) into enhanced cycling, thus favoring their infection, which in turn causes BPA hypersensitivity and eventually leukemic transformation. (3) The leukemia is characterized by prevalent erythropoietic expression, due to the sustained elevation of BPA, via both extrinsic (enhanced release) and intrinsic (increased sensitivity) mechanisms. In FVP animals the erythropoietic component is effective, due to Ep hypersensitivity (up to independence) of infected CFU-E.

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Evidence for the Origin of Hodgkin and Sternberg-Reed Cells from a Newly Detected Small Cell Population

H. Stein, J. Gerdes, U. Schwab, H. Lemke, and V. Diehl

A. Summary

To clarify the origin of Hodgkin (H) and Sternberg-Reed (SR) cells, frozen sections of lymph nodes from 25 patients with Hodgkin's disease were immunostained with a large panel of monoclonal antibodies reactive with cells of lymphoid tissue and granulopoiesis. The results showed that (a) H and SR cells are devoid of markers specific to, or characteristic of B cells, macrophages, dendritic reticulum cells, or interdigitating reticulum cells, and (b) the vast majority of H and SR cells contain granulocyte-related antigens detectable with the monoclonal antibodies TÜ9 and 3C4, but constantly lack other granulocytic cell markers (such as peroxidase and chloroacetate esterase). Monoclonal antibodies raised against a Hodgkin's disease-derived cell line included one, Ki-1, that was found to be selectively reactive with H and SR cells and a minute, but distinct, cell population in normal lymphoid tissue and bone marrow. The latter hitherto unknown cell population appears to be the normal equivalent of H and SR cells.

B. Introduction

Hodgkin's disease is one of the most common types of malignant lymphoma, with approximately 1.5 cases per 100,000 population per year [19]. The Hodgkin (H) and Sternberg-Reed (SR) cells are the most characteristic morphologic elements of the disease. With the recognition and acceptance of H and SR cells as a distinctive neoplastic cell form, there followed a fierce dispute over their derivation. Many diverse theories have been propounded. The more recent ones have related H and SR cells to B cells [1, 4, 7, 10, 11, 20], macrophages [6, 8, 9], dendritic reticulum cells [2], or interdigitating reticulum cells ([5, 12]; Kadin 1981, personal communication).

To test these possibilities, we applied an immunohistologic technique using monoclonal antibodies that are selectively, or nearly selectively, reactive with B cells, macrophages, dendritic reticulum cells, and interdigitating reticulum cells. These studies provided a bulk of negative information, i.e., findings showing the cell types from which H and SR cells probably do not originate.

In an attempt to obtain positive results, we raised monoclonal antibodies against cells of the L428 cell line, which had been shown to share all the investigated conventional immunologic and enzyme markers with H and SR cells [3, 13]. The results of immunostainings with one of the anti-L428 cell hybridoma antibodies suggest that H and SR cells are derived from a unique, as yet unidentified cell population of the lymphoid system.

C. Material and Methods

Fresh unfixed biopsy specimens were obtained from the University Hospitals in Kiel and various other hospitals in northwestern Germany. The tissue was frozen in liquid nitrogen within 12 h of surgical removal.

All the monoclonal antibodies and methods applied in the present study are described in detail elsewhere [14–18].

D. Results and Discussion

The reactivity of H and SR cells of 25 cases of Hodgkin's disease with a large set of cell type-specific or characteristic monoclonal antibodies is shown in Table 1. With the exception of granulocytic cell-reactive antibodies, none of the antibodies reacted with H and SR cells of any case. This is a strong argument against a close relationship between H and SR cells and B cells, macrophages, dendritic reticulum cells, or interdigitating reticulum cells. The reactivity of H and SR cells with antibodies that recognize only cells of granulopoiesis [15] suggests that H and SR cells may be related to cells of the granulocytic series. It appears unlikely that H and SR cells are directly derived from granulopoietic cells, however, because H and SR cells are constantly devoid of peroxidase, chloroacetate esterase, and lysozyme, whereas cells of granulopoiesis constantly express these three markers.

On the whole, the results of our multiple marker analyses clearly show that the antigen and enzyme profile of H and SR cells does not correspond to that of any of the cell types mentioned above. We conclude from these findings that H and SR cells represent a unique cell type that differs from all other cell types identified in the lymphoid system.

This conclusion was substantiated by studies using the Hodgkin's disease-derived cell line L428. First, L428 cells and H and SR cells were immunostained with the same set of monoclonal antibodies. The staining patterns were identical (Table 1).

Table 1. Antigen and enzyme profile of the most important known cell types of the hematopoietic system, cells of the Hodgkin's disease-derived cell line L428, and in situ Hodgkin (H) and Sternberg-Reed (SR) cells of Hodgkin's disease

	B cells	Monocytes macrophages	DRC[a]	IDC[b]	Granulopoietic cells	L428 cells	H and SR cells
SIgM[c]	+	–	+	–	–	–	–
SIgD[c]	+	–	–	–	–	–	–
To15[c] (pan-B cell)	+	–	–	–	–	–	–
C3RTo5[c] (C3b receptor)	+	+	+	–	+/–	–	–/(+)
OKM1[c]	–	+	–/+	–	+	–	–
Monocyte 1[c]	–	+	+	–	–	–	–
Monocyte 2[c]	–	+	–/+	–	–	–	–
TÜ2[c]	–	+	?	–	–/+	–	–
Lysozyme[d]	–	+	–	–	+	–	–
R4/23[c] (dendritic reticulum cells)	–	–	+	–	–	–	–
TÜ9[c,d] (granulocytic cells)	–	–	–/+	–	+	+	+/–
3C4[c,d] (granulocytic cells)	–	–	+/–	–	+	+	+/–
Peroxidase[e]	–	–/(+)	–	–	+	–	–
Chloroacetate esterase[f]	–	–/(+)	–	–	+	–	–
Ki-1[c]	–	–	–	–	–	+	+
Ki-24[c]	–	–	–	–	–	+	+/–
Ki-27[c]	–	–	–	–	–	+	+/–

[a] DRC, dendritic reticulum cells

[b] IDC, interdigitating reticulum cells

[c] Demonstrated by immunoperoxidase staining of frozen sections or acetone-chloroform fixed cytocentrifuge slides

[d] Demonstrated by immunoperoxidase staining of paraffin sections or acetone-chloroform fixed cytocentrifuge slides

[e] Demonstrated by diaminobenzidine staining of frozen sections or unifixed cytocentrifuge slides

[f] Demonstrated by naphthol-AS-D-chloroacetate esterase staining of paraffin sections or methanol-formol fixed cytocentrifuge slides

+ = all cells positive; +/– = some cells positive, other negative; –/(+) = most cells negative and only few cells positive; – = all cells negative

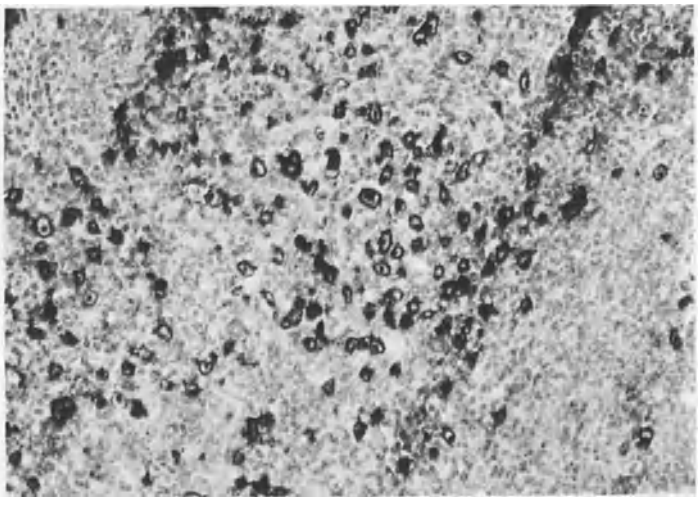

Fig. 1. Frozen section of Hodgkin's disease of mixed cellularity type immunostained with the monoclonal antibody Ki-1. All the Hodgkin and Sternberg-Reed cells are strongly stained, whereas all other cell types failed to react

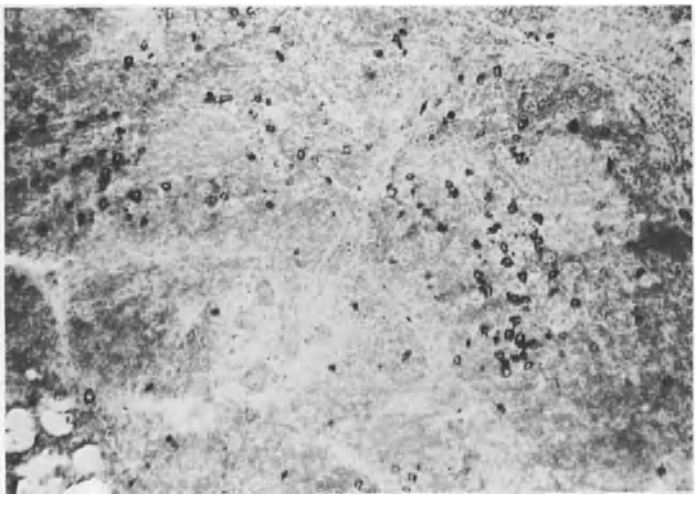

Fig. 2. Frozen section of Piringer's lymphadenitis immunostained with the monoclonal antibody Ki-1. There is a relatively large number of Ki-1 reactive large cells around the germinal centers and between the lymphoid follicles

In a second set of experiments, we raised monoclonal antibodies against the L428 cells and selected the antibodies that reacted with L428 cells, but not with B cells, T cells, macrophages, or B or T region specific reticulum cells. So far, we have obtained three such monoclonal antibodies: Ki-1, Ki-24, and Ki-27. All three antibodies recognize H and SR cells. Ki-24 also reacted with cells of most cases of centroblastic lymphoma, but not with cells in normal lymphoid tissue. Ki-27 recognized not only H and SR cells, but also endothelial cells and smooth muscle cells.

The Ki-1 antibody [14] proved to be the most important and interesting one, because it constantly reacted with H and SR cells (Fig. 1), but not with any of the other known cell types. The nonreactivity of the Ki-1 antibody with cells other than H and SR cells was substantiated by the negative reaction of tumor cells of more than 50 cases of non-Hodgkin's lymphoma of various types and two cases of lysozymet malignant histiocytosis. The Ki-1 antibody also did not react with cells of normal peripheral blood, skin, liver, kidney, lung, or brain, or with macrophages of different types or stages of differentiation.

Unexpected results were obtained, however, when normal and inflamed tonsils and lymph nodes were stained with the Ki-1 antibody using a highly sensitive three-layer immunoperoxidase technique.

A small population of cells located at the outer rim of and between cortical lymphoid follicles was labeled. The distribution of the Ki-1 reactive cells differed from that of all other known cell types. Ki-1+ cells were also detectable, although in small numbers, in the B-cell areas of spleen, the medulla of thymus, and bone marrow.

In cases of certain types of lymphadenitis, e.g., Piringer's lymphadenitis, the number of Ki-1+ cells was found to be remarkably increased (Fig. 2). This finding is of particular interest, because Piringer's lymphadenitis shows some similarities to Hodgkin's disease (e.g., the presence of epithelioid cell clusters and myeloid cells). By staining serial sections of lymphadenitis with increased numbers of Ki-1+ cells, we found that the nonneoplastic cell population expressing Ki-1 antigen did not react with antibodies to B cells, T cells, macrophages, dendritic reticulum cells of the B-cell region, or interdigitating reticulum cells of the T-cell region. These findings suggest that the Ki-1 antibody recognizes a new, as yet unidentified cell population in normal lymphoid tissue. It is tempting to assume that the Ki-1+ cell population is the normal equivalent of H and SR cells.

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Hodgkin's Disease Cell Lines: Characteristics and Biological Activities

V. Diehl, H. Burrichter, M. Schaadt, H. H. Kirchner, C. Fonatsch, H. Stein, J. Gerdes, W. Heit, and A. Ziegler

A. Summary

In the last 4 years we have established five long-term cultures from tumor material of Hodgkin's disease. The in vitro cells have malignant characteristics and represent the in vivo Hodgkin- and Sternberg-Reed-cells as shown by the identity of multiple properties.

Common immunological, functional, and morphological assays did not characterize the in vitro cells as a known cell type of lymphoid, myeloid, or monocytoid tissue. The in vitro Hodgkin's disease cells are biologically active by producing factors involved in regulation and promotion of immunological response and granulopoiesis.

The relevance of the findings for pathogenesis and clinical appearance of Hodgkin's disease is discussed.

B. Introduction

Hodgkin's disease is still one of the most challenging entities in hematooncology. Of patients with this histopathological diagnosis, 60%–70% can possibly be cured [3, 11] and 30%–40% do not respond with complete remission upon first treatment; of this group 15%–20% will achieve remission upon secondary treatment, but 15%–20% will die within 4–18 months in spite of intensive treatment strategies (Fig. 1). Secondary neoplasias are the most hazardous consequence of intensive combined treatment modalities (radiochemotherapy) at the moment and amount to 5%–10% of all initially diagnosed Hodgkin's disease patients [1, 2, 16]. The incidence of acute myeloid or myelomonocytoid leukemia is 130 times higher in Hodgkin's disease patients than in normal individuals [9].

Attempts to investigate the nature of the pathognomonic Hodgkin- (H) and Sternberg-Reed (SR)-cells have been hampered by the fact that these cells constitute only a small minority in the primary biopsy and seem to be utterly growth restricted in the hitherto available culture systems [6].

Since 1978 we have established five cell lines from tumor-involved specimens derived from HD patients [5, 6, 12]. All histologies were confirmed by four independent hematopathologists.

C. Patients, Material, and Methods

All five lines were grown from HD specimens of four patients with nodular sclerosing-type histologies, clinical stage IVB. The patients had been submitted to intensive combination chemoradiotherapy. The sources for the culture material were pleural effusions in three cases and bone marrow and peripheral blood in one case. Two identical cultures were established from the two different sources of this particular patient. Four cell lines continously proliferate in vitro, while on line stopped growing for unknown reasons after 7 months in culture (L 439).

D. Results

The cell lines share an identical (L 428) or partially identical phenotype (L 538/540,

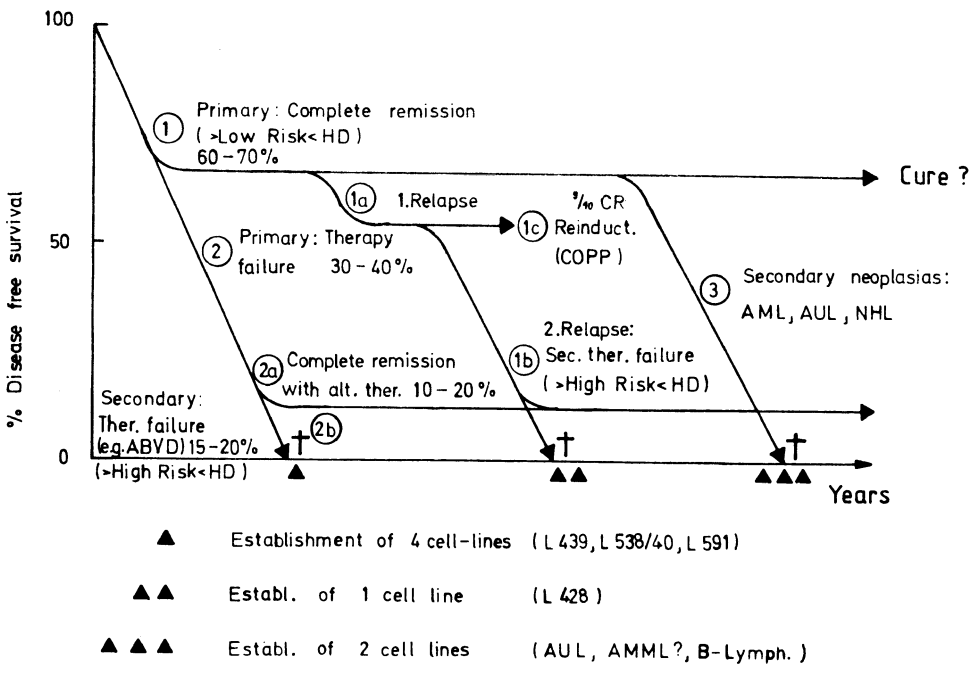

Fig. 1. Hodgkin's disease: clinical course and secondary neoplasias

L 591) with in vivo H- and SR-cells and represent a cell type previously unknown according to the methods used for cellular discrimination [15].

The neoplastic nature of the five HD tumor cell lines is indicated by aneuploidy, except in one line (L 591), and multiple structural and numerical chromosome abnormalities associated with a monoclonal pattern of multiple marker chromosomes.

A comparison of the characteristic features of the in vitro cultured HD cells with H- and SR-cells from freshly obtained biopsies is shown in Table 1. All cultured cells lacked surface- or cytoplasmic-Igs. IgG present in fresh biopsy H- and SR-cells was not found in vitro. Ia-like antigens, receptors for human T cells, acid phosphatase, and acid naphthol acetate esterase were present in all cultured lines. EBV specific receptors were found in two out of two tested lines, EBV genomes and EBV-induced antigens, however, only in one line (L 591). All HD cell lines as well as fresh biopsy H- and SR-cells are devoid of HTLA receptors for C3b, C3d, IgG-Fc, mouse-E, or sheep-E and of lysozyme, peroxidase, and chloracetate esterase (Table 1).

The identity of the in vivo and in vitro H- and SR-cells was shown by congruent morphological, functional, and immunological markers. The strongest proof for the derivation of the cultured cells from H- and SR-cells in vivo was the demonstration of cross-reacting surface and cytoplasmatic constituents on the in vivo and in vitro cells by means of absorbed polyclonal (rabbit anti L 428 cells) (Table 1) and mouse monoclonal (anti L 428 cells) antibodies (Ki 1, Ki 24, Ki 27) (Table 2). Furthermore, monoclonal antibodies directed against granulopoietic cell determinants (3C4, TÜ 9) were also present on biopsy HD cells and the cell lines L 428 KS and L 540, but were absent on the L 591 cells.

In an attempt to determine the origin and nature of the cultured H- and SR-cells a multitude of monoclonal antibodies directed against human lymphoid and hematopoietic differentiation markers were tested against these cells. Table 3 summarizes the results by showing the most commonly accepted markers as specific attributes of the different cell types.

As demonstrated in this table, the reactive pattern of the cell line L 428 and partially of the cell line L 540 was identical

Table 1. Properties of in vivo and in vitro H- and SR-cells

	HD and SR cells in biopsies	HD cell lines			
		428	439	538/540	591
Surface staining for					
IgG, IgM, IgA	–	–	–	–	–
Lysozyme	–	–	–	–	
Ia-like antigen	+	+	+	+	+
Cytoplasmic staining for					
IgG	+	–	–	–	–
Lysozyme	–	–	–	–	–
Rosette assays with					
EAC 3b, EAC 3d	–	–	–	–	60% – 70%
IgG – EA	–	–	–	–	–
Human T cells	+	+	+	–	+
Immunphagocytosis					
C-3B-coated E, IgG-coated E	–	–	–	–	–
EBV-specific antigens					
EBV-receptors	N.T.	+	N.T.	N.T.	+
EBNA, EA, VCA	N.T.	–	–	–	+
Cytochemical staining					
Naphthol chloracetate esterase, Peroxidase Alkaline phosphatase	–	–	–	–	–
Acid-α-naphthyl acetate esterase Acid phosphatase	+	+	+	+	+
Reactivity with heterologous L 428 antiserum	+	+	+	+	+

with that of the H- and SR-biopsy cells, lacking markers characterizing B cells, T cells, monocytes, dendritic reticulum cells, interdigitating reticulum cells, and Null cells. They carried, however, granulopoietic cell determinants, as shown by the reactivity of antibodies 3C4, TÜ 9, and Vim D5, but they were unreactive for the peroxidase and chloracetate esterase cytochemical staining.

The L 540 cells reacted like the L 428 cells, but were positive with the monocyte 1 antibody. The L 591 cells showed a very peculiar pattern insofar as they lacked

HD material	Antibodies				
	L 428 Antibodies			Granulopoietic cell antibodies	
	KI 1	KI 27	KI 24	3C4	TÜ 9
HD biopsies	+	+	+	+	+
HD cell lines					
L 428	+	+	+	+	+
L 428 KS	+	+	+	+	+
L 428 KSA	+	–	+	N.T.	N.T.
L 540	+	–	–	+	+
L 591	+	+	+	–	–

Table 2. Reactivity of HD and SR cells in biopsies and in vitro (lines) with monoclonal L 428- and granulopoietic cell antibodies

Table 3. Differential characteristics of HD and SR cells and HD cell lines

Cell Type	Markers/monoclonal Antibodies	H- and SR-cells in Biopsies	HD-derived cell lines		
			L 428	L 540	L 591
B cells	Anti-IgM-D	–	–	–	–
	Ig surface	–	–	–	–
	Ig cytoplasm	–	–	–	–
	EBV antigens	–	–	–	+
T cells	OKT_{11}	–	–	–	+
	LYT_3	–	–	–	+
Monocytes	OKM_1	–	–	–	–
	$Monocyte_1$	–	–	+	–
	$Monocyte_2$	–	–	–	–
	Lysozyme production	–	–	–	–
	Phagocytosis	–	–	–	–
Granulopoietic cells	3C4	+	+	+	–
	TÜ 9	+	+	+	–
	VIM-D5	+	+	N.T.	N.T.
	Peroxidase	–	–	–	–
	Chloracetate esterase	–	–	–	–
Dendritic reticulum cells	R 4/23	–	–	–	–
Interdigitating reticulum cells	$NA_{1/34}$	–	–	–	–
	$T\text{-}ALL_2$	–	–	–	–
Null cells (NK cells?)	OKM_1	–	–	–	–
HD-derived cells (H- and SR-cells)	Ki 1 } anti 428 cells	+	+	+	+
	Ki 24 } anti 428 cells	+	+	–	–
	Ki 27 } anti 428 cells	+	+	–	–
	Heterologous anti 428 serum	+	+	+	+

Table 4. Biological activities of HD line supernatants

	HD cell lines					Control lines	
	L 428	L 428 KS	L 428 KSA	L 540/538	591	LCL	BL lines RAMOS, HRIK
Granulocyte colony stimulating activity (CSA)	+	+ +	+ + +	+ +	(+)	0	0
Interleukin 1 activity	+	+	+	+	+	0	0
Accessory function for mitogen-induced T-cell proliferation	+	+	+	N.T.	N.T.	0	0
Stimulation of mixed lymphocyte reaction	+	+	N.T.	N.T.	N.T.	0	0
Enhancement of EBV-induced spontaneous B-cell transformation	0	0	+	0	0	–	–

B-cell markers like Ig production or Ig surface reactivity (anti IgM, anti IgD), but carried C3b and C3d receptors, as well as EBV receptors and EBV antigens. Furthermore, they reacted positively with T-cell antisera like OKT-11 and Lyt 13, but lacked monocyte, granulopoietic cell, dendritic reticulum cell, and interdigitating reticulum cell, as well as Null cell markers.

The cell lines produced a variety of substances which are known to be mediators of immune response and granulopoiesis (Table 4):

Conditioned media of all cell lines contained high amounts of granulocyte colony stimulating activity [6]. Furthermore, supernatants of all HD-derived cell lines showed pronounced Interleukin 1 activity. The L 428 line and its sublines (for origin see [6]) exhibited an accessory function for mitogen (CON-A)-induced T-cell proliferation, as well as a stimulation of mixed lymphocyte reaction [7, 8]. Conditioned medium of the TPA-treated L 428 KSA adherent subline [6] enhanced the EBV-induced B-cell transformation to immortalized, continously growing lymphoblastoid cell cultures, when using EBV-positive healthy donors.

E. Conclusions

1. L 428 respresents in vitro the in vivo H- and SR-cell population. The other reported lines exhibit most but not all markers of in vivo H- and SR-cells. The non-LCL character of L 591 is still the subject of discussion.

2. The HD cells (in vivo and in vitro) represent no known cell type or any cell class so far identified by common immunological, functional, and morphological tests.

3. The in vitro HD cells exhibit biological activities regulating and/or promoting immune response and granulopoiesis.

F. Hypothetic Pathogenesis of Hodgkin's Disease

Figure 2 summarizes all the data obtained on the described HD-derived cell lines in an attempt to propose a hypothesis for some of the pathogenetic mechanisms involved in Hodgkin's disease:

The origin of H- and SR-cells in unknown. It is possible that the Ki 1 antibody not only recognizes H-SR-specific determinants, but also depicts a previously

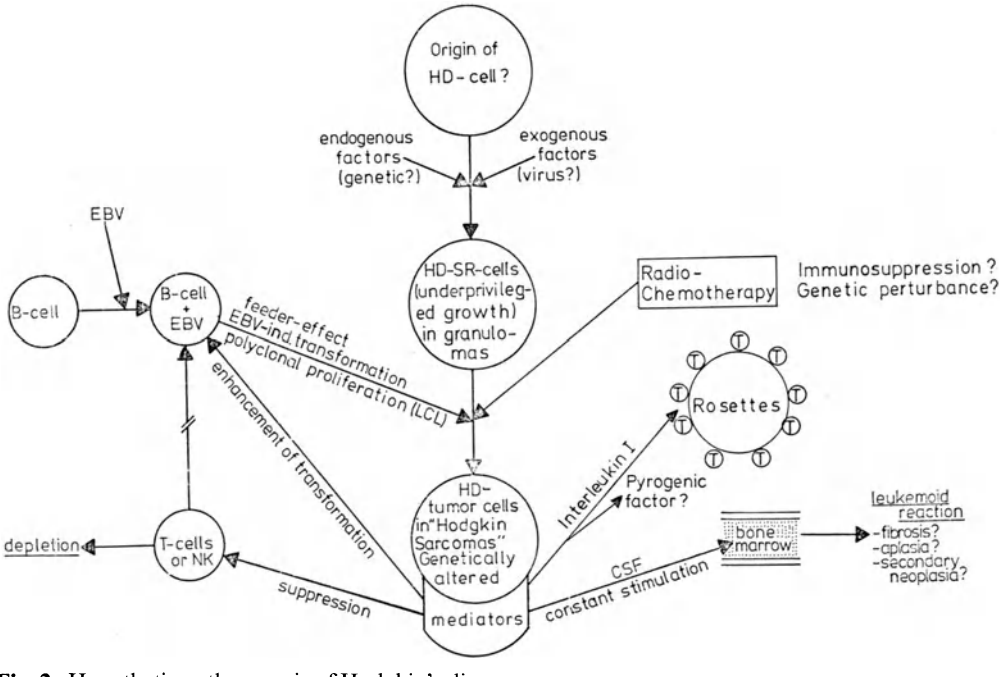

Fig. 2. Hypothetic pathogenesis of Hodgkin's disease

undefined cell in normal tissue [13, 15], which could be the normal counterpart of the "malignant" H- and SR-cells (see Stein, this volume).

The pathogenetic mechanisms involved in the transformation of a normal cell, possibly playing some role in immune and hematopoietic regulation, is unknown. Endogenous (genetic?) and exogenous (viruses, chemical agents, both?) might induce a gradual "evolution" from a primarily nonproliferating, biologically active cell, which by its products (CSF, Il 1) might create the clinically not very aggressive "Hodgkin's lymphoma", to a genetically altered (Fonatsch et al. unpublished results) more malignant cell, embedded in the histological entity of a "Hodgkin sarcoma." Radiochemotherapy might act as a cofactor in this process of gradual malignization. Of the HD patients, however, 60%–90% are cured by radio- and/or chemotherapy in the early stages of this process before genetically altered cells have chance to commence rapid proliferation and possibly exert resistance to cytoreductive therapy.

The variance in the histological presentation of Hodgkin's disease could reflect this gradual malignization process: Paragranuloma and/or lymphocytic predominance and lymphocyte-enriched nodular sclerosis would identify a stage of "low risk", with a high functional activity of the H- and SR-cells, producing mediators like CSF, Interleukin 1, but still restricted in cellular proliferation. If cytoreductive therapy is carried out at this stage, cure is possible in up to 90% of cases ([10], Schellong, personal communication).

If the HD cells withstand therapy by either genetically inherent or resistance mechanisms acquired during treatment, the patient will present a picture of a more malignant Hodgkin's sarcoma with a higher number of rapidly proliferating H- and SR-cells. These cells could still have retained their biological mediator production, but the balance might be toward more production of immune suppressive and EBV transformation enhancing factor.

The fact that many Hodgkin's disease patients develop high antibody serum titers against EBV antigens and give rise to EBV-induced lymphoblastoid cell cultures significantly more than normal individuals [4] could be explained not only by T-cell immunosuppression but also by a direct influence of an EBV transformation enhancing factor. The resulting polyclonal lymphoblastoid transformation could "feed" or protect the tumor cell, possibly under a concomitant protection of the rosetting OKT-4-positive T-helper cells, attaching to the H- and SR-cells. These protection mechanisms might enable an a priori "low-grade malignant" HD cell to "sneak through" to a higher malignant proliferating tumor cell, which in 15%–20% of the clinical outcome could eventually kill the patient. Most Hodkgin's disease patients, however, do not die of tumor cell proliferation, but of biological side effects of immune deficiency and hematological complications, possibly due to some of the described factors.

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Prelymphoma, Early Lymphoma, and Manifest Lymphoma in Immunosialadenitis (Sjögren's Syndrome) – A Model of Lymphomagenesis

K. Lennert and U. Schmid

A. Summary

The development of malignant lymphoma in myoepithelial sialadenitis with and without Sjögren's syndrome was investigated. At first, prelymphomatous proliferation areas showing a polytypic immunoglobulin (Ig) pattern were seen. These transformed into malignant lymphoma with a monotypic Ig pattern, at first in small, circumscribed proliferation areas ("early lymphoma") and later in large, confluent proliferation areas ("manifest lymphoma"). The lymphomas were classified as LP immunocytoma. In some cases they transformed into high-grade malignant lymphomas of the same category, namely, B-immunoblastic lymphoma. The same types of lymphoma have been found in NZB mice and chronic graft-versus-host reactions. "Primary" malignant lymphomas of salivary glands that did not show myoepithelial sialadenitis were also studied. These lymphomas were mostly germinal center cell tumors and probably developed primarily in lymph nodes within parotid glands in most, if not all, cases.

B. Introduction

In 1964, Talal and Bunim [8] recognized that there is a high frequency of malignant lymphoma among patients with Sjögren's syndrome. Later, Anderson and Talal [1] reported that the cells of the "histiocytic" lymphomas developing in Sjögren's syndrome showed a monoclonal immunoglobulin (Ig) pattern, usually IgM/$\varkappa$. Such lymphomas thus have to be interpreted as B-immunoblastic lymphoma. In the same article, Anderson and Talal complained that their pathologist and some consultants were not able to diagnose a fair number of cases that showed monoclonal IgM/$\varkappa$, but did not exhibit an immunoblastic morphology. We speculate that those tumors were lymphoplasmacytic/lymphoplasmacytoid lymphomas (LP immunocytoma).

We also found it very difficult to interpret lymphoid infiltration of salivary glands. Thus new techniques have been applied to the cases in our collection. In particular, the immunoperoxidase method was used to demonstrate cytoplasmic Ig and lysozyme. Follow-up studies were also performed to learn more about the clinical picture and outcome. The latter investigations were initially done with the assistance of Drs. M. Burkert (Kiel), Renate Reif (Zrifin, Israel), and Dagmar Helbron (Kiel). Recently, we examined the material collected at the Lymph Node Registry in Kiel with regard to the following lesions:

1. Myoepithelial sialadenitis (MESA) with and without Sjögren's syndrome
2. So-called primary malignant lymphoma of salivary glands
3. Unclear lymphoid lesions of salivary glands.

C. Material and Methods

Paraffin sections from a total of 72 cases were stained with Giemsa, silver impregnation (Gomori), periodic acid Schiff (PAS), and hematoxylin and eosin (H & E).

	With MESA	Without MESA
ML lymphoplasmacytic/-cytoid (LP immunocytoma)	23 (3[a])	2
ML immunoblastic (B)	3 (3[a])	2
ML centroblastic-centrocytic	–	15
ML centroblastic	–	2
Hodgkin's disease	–	4
Total	26 (+6[a])	25

[a] Cases showing only extrasalivary ML

Table 1. Types of malignant lymphoma (ML) diagnosed on salivary gland biopsies with and without myoepithelial sialadenitis (MESA)

A modification [3] of the immunoperoxidase method of Sternberger et al. [6] was used to demonstrate α, γ, μ, J, $\varkappa$, and λ chains and lysozyme as described in detail elsewhere [4].

D. Results and Discussion

Myoepithelial islands are the essential feature of the sialadenitis seen in Sjögren's syndrome. There are isomorphic cases of sialadenitis, however, without the clinical features of Sjögren's syndrome. Thus we use the term "myoepithelial sialadenitis" (MESA) to designate all cases showing myoepithelial proliferation and lymphoid infiltration of salivary glands. MESA was chosen as a distinguishing criterion for the present study. We simply separated lymphoid proliferation or malignant lymphomas with MESA and those without MESA.

The types of malignant lymphoma diagnosed on salivary gland biopsies are shown in Table 1. There was a significant difference in the occurrence of the various types of malignant lymphoma between the groups with and without MESA. Patients with MESA showed only LP immunocytoma or its high-grade malignant variant, B-immunoblastic lymphoma. In contrast, only a few of the patients without MESA had these types of lymphoma, while a majority showed germinal center cell tumors.

Table 2. Results of immunoperoxidase (PAP) staining and occurrence of extrasalivary lymphoma in 45 patients with myoepithelial sialadenitis (MESA)

	n	PAP	Extrasalivary lymphoma	
			with MESA	later (10 – 66 months)
MESA without proliferation areas	3			
Polytypic		3	–	–
MESA with small, circumscribed proliferation areas	16			
Polytypic		4	–	–
Monotypic		9	–	3
Not studied		3	–	1
MESA with extensive, confluent proliferation areas	26			
Polytypic		–	–	–
Monotypic		18	6	2
Not studied		8	2	4

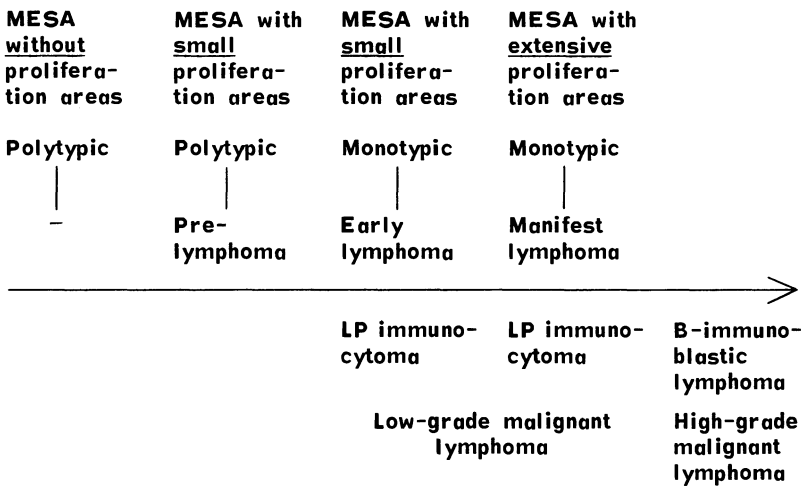

Fig. 1. Schematic diagram of development of malignant lymphoma in myoepithelial sialadenitis (MESA)

There were also four cases of Hodgkin's disease in the group without MESA and none in the group with MESA.

A remarkable finding in the cases without MESA was the very frequent presence of residual lymph node tissue. This indicates that most, if not all, of the malignant lymphomas developed in lymph nodes within salivary glands (specifically, parotid glands) and not in the salivary glands themselves. In contrast, we did not find residual lymph node tissue in any of the cases with MESA and thus assume that the lymphomas truly developed primarily in salivary glands.

An analysis of all our cases histologically characterized by MESA (see Table 2) revealed that the myoepithelial complexes were sometimes surrounded by uniformly dense infiltrates of lymphocytes with a few plasma cells; this pattern was found in three cases. A larger number of cases (16), however, showed small, circumscribed proliferation areas, while a majority (26) of the cases showed large, confluent proliferation areas. The proliferation areas contained large immunoblasts, a few medium-sized lymphoid cells, and plasmacytoid cells, and they often exhibited mitotic activity.

With immunoperoxidase staining, the plasmacytoid cells of MESA without proliferation areas showed a polytypic Ig pattern. The MESA group with small proliferation areas contained both polytypic and monotypic cases. In the cases of MESA with large proliferation areas, the Ig pattern of the lymphoplasmacytoid proliferation areas was exclusively monotypic.

A malignant lymphoma of the same type as the salivary gland lymphoma sometimes developed in extrasalivary tissue. This occurred at the same time as MESA only in the cases with large proliferation areas, whereas later development of extrasalivary lymphoma was also seen in cases with small proliferation areas (see Table 2).

Assuming that a monotypic Ig pattern is solid evidence of malignant proliferation, it is possible to sketch the development of malignant lymphoma in MESA (Fig. 1). At first, MESA does not show proliferation areas and contains only plasma cells with a polytypic Ig pattern. Later, small proliferation areas showing a polytypic Ig pattern appear in the diffuse lymphoid infiltrates; we interpret this lesion as a prelymphoma. Finally, the Ig pattern becomes monotypic, and the small proliferation areas probably represent an early lymphoma. Such cases do not show any extrasalivary manifestation. After a latency period of 10–66 months, however, extrasalivary lymphoid proliferation is seen, and it shows the same morphology as the salivary gland, namely, that of LP immunocytoma.

MESA with extensive, confluent proliferation areas always has a monotypic Ig pattern and can probably be interpreted as

manifest malignant lymphoma (specifically, LP immunocytoma). This view is supported by the simultaneous presence of a malignant lymphoma with the same morphology (LP immunocytoma) outside the salivary gland in some cases.

In a few cases, the LP immunocytoma, especially if it was extrasalivary, was observed to transform into B-immunoblastic lymphoma. Since this lymphoma type is the high-grade malignant variant of LP immunocytoma, the final stage of development has been reached. As outlined in Fig. 1, an initially benign proliferation ("prelymphoma"; small proliferation areas, polytypic Ig pattern) leads to an early lymphoma (small proliferation areas, monotypic Ig pattern), which develops into a manifest lymphoma (large proliferation areas, monotypic Ig pattern). The lymphoma is always LP immunocytoma, which is a low-grade malignant lymphoma according to our classification. This low-grade malignant lymphoma may transform into a high-grade malignant lymphoma of the same cell series, namely, B-immunoblastic lymphoma.

It is not possible to determine the incidence of such lymphoma development, because the material collected at the Lymph Node Registry is highly selected.

Analysis of the Ig classes in the immunoblasts and plasmacytoid cells of the monotypic cases revealed a marked predominance of IgM/$\varkappa$. There were also a few cases with IgM/λ, IgG/$\varkappa$, or IgA/λ.

It is not always possible to determine from the morphology alone whether a proliferation has a monotypic or polytypic Ig pattern. Nevertheless, the following criteria were found to be of some help: epithelioid cells and intranuclear PAS-positive inclusions occurred only in proliferation areas with a monotypic Ig pattern, and giant cells of Sternberg-Reed type were seen almost only in such areas.

In order to interpret our findings, it may be helpful to compare them with reports on similar lesions in animals. MESA is now considered to be an immunosialadenitis and is placed in the group of autoimmune disorders. A similar type of sialadenitis has been seen in NZB mice [7, 9] that spontaneously developed an autoimmune disease comparable to Sjögren's syndrome. Similar lesions have also been found by Gleichmann et al. [2] in salivary glands of mice with chronic graft-versus-host reactions and by Shulman et al. [5] in chronic graft-versus-host syndrome in man. Moreover, in NZB mice and mice with chronic graft-versus-host reactions, the same types of malignant lymphoma develop as in MESA, namely, LP immunocytoma with macroglobulinemia and B-immunoblastic lymphoma.

Because of the types of malignant lymphoma found in chronic graft-versus-host reactions and the similarity in pathogenesis between autoimmune disorders and chronic graft-versus-host syndrome, we may speculate that the basic mechanisms behind the development of malignant lymphoma in both diseases are the same. According to Gleichmann et al. [2], this mechanism is produced by histoincompatibility between B cells, which might be virus infected, and the controlling T cells, which stimulate the "not self"-looking B cells to proliferate. It has not yet been determined whether this T-cell stimulation is enough for the final development of a malignant B-cell proliferation, or whether a second factor is necessary.

The development of LP immunocytoma and B-immunoblastic lymphoma in a defined experimental and immunologic situation not only provides indications regarding their developmental mechanism; it also shows that LP immunocytoma and B-immunoblastic lymphoma are clearly identifiable entities. Indeed, we must differentiate them in order to gain insight into the genesis of malignant lymphomas. Placing these entities in the same categories with other lymphoma types [as in the Working Formulation (10)] would obscure pathogenetic relationships.

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B-Cell Malignancies: Origin and Extent of Clonal Involvement *

M. D. Cooper and H. Kubagawa

Soon after it was established that normal lymphoid development proceeds along two distinct pathways of differentiation, it was recognized that lymphoid malignancies affected cells of either T or B lineage, and not both [1]. Studies conducted with animal models of lymphoblastic leukemias and lymphomas revealed that malignant T and B cells, like their normal counterparts, have their origin in central lymphoid tissues. The thymus is essential in the genesis of a variety of murine lymphoid malignancies that are induced by oncogenic viruses, ionizing irradiation, carcinogenic hydrocarbons, and hormones, or that arise spontaneously in AKR mice (reviewed [2]). Removal of the thymus prevents these lymphoid malignancies, and thymus transplants restore susceptibility [2–4]. This is due to an initial transformation of thymocytes with subsequent seeding or metastasis to peripheral tissues. On the other hand, the bursa of Fabricius is the source of malignant B cells in avian lymphoid leukosis [5, 6].

Avian lymphoid leukosis was the first model of a virus-induced B-cell malignancy and several of its features are relevant to the analysis of human B-cell malignancies. This B-cell lymphoma can be induced by infection of embryos or newly hatched chicks with avian group A leukemia retroviruses [7, 8]. The virus infects many cell types, but it selectively transforms B cells [6, 9]. Moreover, the virus-induced transformation only occurs at a very early stage in B-cell differentiation within the inductive bursal microenvironment. There are two distinctive phases in the evolution of this virus-induced malignancy of selected B-cell clones. First, one or more of the thousands of lymphoid follicles within the bursa exhibit lymphoblastic transformation. The transformed follicles are evident within 1–2 months after virus infection at hatching. The next phase usually occurs between 5 and 9 months of age, and involves widespread seeding and malignant growth of B cells, most of which do not become mature plasma cells. Bursectomy or physiological bursal regression prior to this second stage will abort the fatal B-cell malignancy [5, 6].

In the lymphoma cells, viral promoter sequences have been found to be integrated with a cellular *onc* gene called c-*myc* [10]. The activation of this transforming gene may be responsible for the initial transformation of lymphoid cells in the bursal follicles. However, the activated c-*myc* gene is unrelated to the transforming gene that has been detected by transfection with lymphoma DNA [11]. Activation of the latter *onc* gene could be responsible for the second step in the evolution of a malignant B-cell clone. Another hypothesis is that antigen-induced growth of transformed B cells may play a significant role in the malignant lymphomatosis phase [9]. The retrovirus itself could serve as the stimulating antigen for B-cell clones with appropriate immunoglobulin receptors [12].

We hypothesized that human B-cell malignancies would also involve an initial transformation of B-cell clones within the inductive microenvironment and that antigens could influence the subsequent be-

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havior of the affected clones. In this chapter we review the results of our studies on a spectrum of human B-cell malignancies within the context of normal B-cell differentiation. To identify the affected B cells we have used the immunoglobulins which they produce as clonal markers. Antibodies were prepared against immunoglobulin heavy and light chain isotypes, V_H subgroups and idiotypes, and these were used to diagnose and analyze pre-B leukemias, B-cell leukemias, Waldenström's macroglobulinemia, and multiple myelomas.

A. Normal B-Cell Differentiation

Cells of B lineage are unique in their expression of the immunoglobulin genes, and progression along this differentiation pathway can be discerned by determining which immunoglobulin genes are expressed. Immunoglobulin molecules consist of identical pairs of heavy and light chains. In the mouse, these are encoded by linked families of V_H, D_H, and J_H genes located 5′ to the heavy chain constant region (C_H) genes, the order of which is μ, δ, γ_3, γ_1, γ_{2b}, γ_{2a}, ε, and α on chromosome 12 [13–15]. The V_L and J_L genes are upstream from the $\varkappa$ and λ light chain genes on chromosomes 6 and 16, respectively [14, 16, 17]. The corresponding *H*, $\varkappa$, and λ immunoglobulin gene families in human are located on chromosomes 14, 2, and 22 [18–21].

One of the first steps in differentiation is the assembly by chromosomal rearrangement of one each of the V_H, D_H, and J_H genes and the transcription of the *V-D-J* set along with the *C*μ gene [22, 23]. A cell expressing such μRNA is known as a pre-B cell, and at this stage few of the μ chains reach the cell surface [24–29]. Next, one set of V_L and J_L genes is productively rearranged and a complete IgM molecule is expressed on the cell surface. This differentiation event marks the birth of an immature B-lymphocyte, and the point at which antigens may begin to influence the cell's behavior.

The foregoing stages, stem cell → pre-B → immature B, occur initially within inductive microenvironments of the fetal liver and thereafter in the bone marrow. Subsequent stages in B-cell differentiation entail changes in the expression of the C_H genes, without alteration in expression of the V_H–D_H–J_H set or the light chain gene. Intermediate stages in B-lymphocyte differentiation are marked by the expression of a variety of cell surface proteins involved in regulating migration, growth, and differentiation of B cells into terminally differentiated plasma cells with the associated shift from surface expression to secretion of immunoglobulin molecules.

Immature B cells first express surface IgM and later coexpress IgD with the same V_H–D_H–J_H and light chains. Some members within each B cell clone undergo a further switch, from IgM (and IgD) to IgG, IgA, or IgE [30], and all members of the clone will of course share the same antibody specificity and idiotype. Current views on heavy chain isotype switching mechanisms, sequence, and regulation are reviewed elsewhere [31].

The number of B-cell clones within an individual is very large, probably well over a million. Each expresses a unique antibody specificity and idiotypic pattern, but may share cross-reactive idiotypes with other clones [32].

B. Pre-B Leukemias

Approximately 20% of all acute lymphocytic leukemias of childhood can be recognized as pre-B leukemias by the presence of intracytoplasmic μ chains, absence of surface immunoglobulin (Ig), characteristic lymphoid morphology with lobulated nucleus and marrow cytoplasmic rim, surface expression of B-cell differentiation antigens, and the absence of T-cell and myelomonocytic antigen markers [33–36]. Another large segment of acute lymphocytic leukemias, perhaps 50%–60%, can be recognized as "pre" pre-B cells by detecting rearrangements of immunoglobulin heavy chain genes [28] and the expression of B-cell surface antigens [37–39]. Other characteristic features are the expression of HLA-DR, common ALL antigen, and terminal deoxynucleotidyl transferase activity [33–36].

There is suggestive evidence that the more differentiated μ^+ pre-B leukemias have a worse prognosis than the μ^- pre-B

leukemias [40]. Neither follows the relentless and rapid downhill course of the childhood B-cell leukemias, which are featured by surface IgM expression.

The target cell for the oncogenic events appears to be an Ig^- bone marrow precursor cell. Even in the μ^+ pre-B leukemias, some members of the leukemic clone do not express μ chains. More compelling evidence comes from the study of individuals with chronic myelogenous leukemia. Analysis of chromosomal markers (i.e., the Philadelphia chromosomal aberration on chromosome 22 and the G6PD isoenzymes, or alleles, encoded on the X chromosomes) has revealed that normal blood cells in these patients are derived from the same pluripotent stem cell as the myelogenous leukemia cells [41, 42]. More relevant to our theme here are the patients who undergo conversion from chronic myelogenous leukemia to acute lymphocytic leukemia of pre-B phenotype. Chromosomal marker analysis indicates that both lines of malignant cells are sequentially derived from the same multipotent stem cells [43–45].

The patterns of immunoglobulin gene expression in pre-B leukemia clones are also informative. Most pre-B leukemia cells express μ chains but no light chains, a finding that is consistent with the asynchronous onset of heavy and light chain expression observed in normal pre-B cells [46–50]. Unlike normal pre-B cells, however, subpopulations of pre-B cells within the leukemic clones may express heavy chain isotypes other than μ [33, 35]. In order to examine further the heavy chain isotope switching in leukemic pre-B cells, we have used monoclonal antibodies in immunofluorescence assays to allow unambiguous assignment of the heavy chain isotopes expressed by individual leukemic cells. Switching in leukemic pre-B clones from 11 childhood leukemias invariably led to expression of γ_1 heavy chains, and less often to expression of γ_4 and α [51]. The observed frequencies of isotype switches, μ to $\gamma_1 > \gamma_4 \gg \alpha$ and the absence of δ, γ_2, γ_3, and ε, indicate a preferential order for the switching process in leukemic pre-B cell clones. Since these cells lack surface immunoglobulins, these data favor a stochastic model for isotype switching rather than an antigen-induced switch mechanism.

So far the order of human C_H genes on chromosome 14 [18] has only been partially elucidated. Cμ and $C\delta$ are thought to be next in line 3′ to the J_H genes as is the case in mice [52]. $C\gamma_2$ is 5′ to $C\gamma_4$ [53, 54]; $C\gamma_1$ appears to be 5′ to $C\gamma_3$ [54]; and $C\varepsilon$ genes are thought to be located 5′ to the $C\alpha_1$ and $C\alpha_2$ gene [55]. Although our observations would fit with a gene order in man of μ, δ, γ_1, γ_3, γ_2, γ_4, ε, and α, the data indicate that the switch sequence cannot be explained solely by the C_H gene order.

The results of two-color immunofluorescence analysis indicated that individual pre-B cells within the leukemic clones express as many as three or even four heavy chain isotypes [51]. The stability of these phenotypic patterns has not been examined yet, but the presence of multiple heavy chain isotypes in individual pre-B cells might be explained by the hypothesis of a preliminary switch mechanism involving a large primary transcript of all the C_H genes and differential RNA splicing [56, 57]. This hypothesis does not, however, simplify the problem of ordered switching in the leukemic pre-B cells.

Another remarkable finding in our studies was the expression of $\varkappa$ light chains by almost all of the leukemic pre-B cells exhibiting heavy chain switches. This preference for $\varkappa$ over λ expression might be expected in view of evidence which suggests that $V\lambda$–$J\lambda$ gene rearrangement for expression with $C\lambda$ regularly follow nonproductive rearrangements of $\varkappa$ genes on both chromosomes [58–60]. However, the consistent acquisition of a productive $\varkappa$ gene by switching pre-B cells is unprecedented and suggests that these genetic events, occurring on chromosomes 14 and 2 [18–20], are coupled by a regulatory mechanism that remains to be elucidated.

Occasional clones of leukemic pre-B cells appear to continue differentiation into B-lymphocytes. One such example is il-

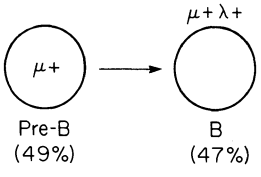

Fig. 1. Cell phenotypes in the transitional form of acute lymphocytic leukemia

lustrated in Fig. 1. Approximately half of the leukemic cells in this patient were μ^+ pre-B cells and the other half IgMλ-bearing B-lymphocytes. This suggests that the transformation process per se does not necessarily preclude continued differentiation beyond the pre-B compartment, and this principle has been confirmed in the following studies of B-cell leukemias and multiple myelomas.

C. B-Cell Leukemias

B-cell leukemias are monoclonal lymphoproliferative disorders marked by the expression of surface immunoglobulin (reviewed in [61]). Most of them express surface IgM, or IgM and IgD together. Less frequently the leukemic B cells express IgG or IgA. B-cell leukemias are closely related to B-cell lymphomas; their distinction rests primarily upon the predominant migration pattern of the involved B-cell clone, i.e., lymphoid tissues versus circulation. The affected B cells usually fail to differentiate into mature plasma cells, although some malignant B-cell clones contain mature antibody-secreting members, and many can be induced to differentiate into plasma cells *in vitro* [62–64].

An ideal marker for malignant B cells is the idiotype (Id) of the immunoglobulin that they express. Anti-Id antibodies have been difficult to prepare for B-cell leukemias and lymphomas, because they produce so little of their immunoglobulin product. However, hybridoma technology now makes it feasible to make monoclonal antibodies to the Id determinants expressed by malignant B-cell clones. We have prepared monoclonal antibodies specific for the Id determinants on leukemic B cells from selected patients, and have used these anti-Id antibodies to trace the extent of clonal involvement.

Ninety percent of the circulating mononuclear cells (18,000/mm^3) in one such patient were small lymphocytes bearing IgMλ and IgDλ molecules. Virtually all of these were reactive with a monoclonal anti-Id antibody tailor made against her leukemic B cells [65]. IgG and IgA B cells were very rare in this woman but, of these, 40% and 25% were reactive with the same monoclonal anti-Id antibody. This suggests that a few members of the leukemic clone have undergone heavy isotype switches, the frequency of which was governed by rate-limiting feature of the switch process.

Additional information can be obtained by study of bone marrow in addition to the blood cells in individuals with B-cell leukemia. This is illustrated by our findings in an elderly man with acute lymphoblastic leukemia cells that expressed surface IgA$_1\varkappa$ molecules [66]. All of his leukemic cells were reactive with one of a panel of four monoclonal anti-V_H subgroup antibodies [67]; a monoclonal anti-Id antibody (WF) was prepared which reacted with all of the leukemic IgA B cells and $<1\%$ of normal B cells. None of the plasma cells found in this patient expressed the homologous idiotype, suggesting that the leukemic B-lymphocytes did not complete differentiation. The expression of the WF idiotype by cells in the circulation was restricted to the IgA$_1\varkappa$ leukemic B cells; no T cells or IgM and IgG B cells with the WF Id could be found.

The picture was different in the bone marrow of this patient (see Fig. 2). Here we found a small subpopulation of IgM$\varkappa$ lymphoblasts that expressed the WF idiotype and the same V_H subgroup as the IgA$_1\varkappa$ lymphoblasts. Pre-B cells containing α, γ, and μ chains of the same V_H subgroup were also present in the bone marrow. The lineal relationship between the μ, γ, and α pre-B cells was indicated by presence of both μ and γ, and of γ and α together in some of these pre-B cells. Light chain expression was not evident in these pre-B cells; this precluded identification with the monoclonal anti-Id antibody, because it recognized an idiotope formed by the heavy and light chains combined. When the bone marrow sample was depleted of B-lymphocytes and placed in culture, IgA$_1\varkappa$ B cells with the WF Id were generated. These results suggest that this leukemic clone was transformed prior to the heavy chain switch and before $\varkappa$ light chain expression. The basis for the preferential expansion of the IgA$_1$ B-cell subpopulation is unclear. These cells did not display translocations on chromosomes 14 or 2 as have been observed in IgM$\varkappa$ Burkitt's lymphomas [21, 68]. It is noteworthy, however, that examination of

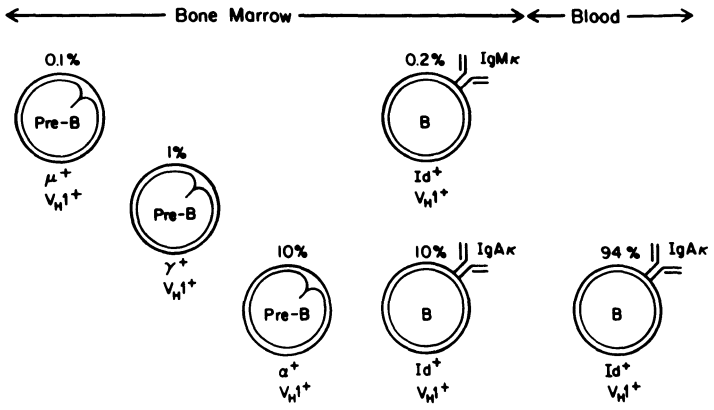

Fig. 2. Extent of clonal involvement in patient W.F. with acute lymphocytic leukemia

the DNA from the IgA_1 leukemic cells failed to reveal deletion of all of the γ genes (J. Ellison, unpublished). Deletion of C_H genes 5′ to the expressed C_H gene on one or both chromosomes has been a consistent feature in mouse myeloma cells, but this would appear to be the first attempt to examine this switch event at the B-lymphocyte level.

D. Plasma Cell Malignancies

Multiple myeloma is a B-cell malignancy that has classically been thought to involve bone marrow plasma cells. This viewpoint has been modified by the demonstration of an increase in circulating B-lymphocytes bearing the homologous idiotype. In two patients with IgA myelomas, we found expression of the homologous Id on IgM^+/IgD^+ B-lymphocytes as well as on IgA B-lymphocyte precursors. Moreover, a few Id^+ cells of the μ^+ pre-B phenotype were identified in the bone marrow [69]. Similar observations were made in studies of a patient with an IgD myeloma [65]. Hence, we have proposed that even multiple myelomas have roots within the pre-B cell compartment, and the bone marrow predilection of the myeloma population may be due to its genesis from marrow stem cells.

In a woman with Waldenström's macroglobulinemia, most of the circulating B cells had surface IgM with the homologous idiotype. In addition, 25% of her circulating IgA B cells expressed the same idiotype, suggesting that these cells belonged to the malignant clone as well [65]. The IgA^+ members of the clone were different from their sister IgM cells in that they apparently did not complete differentiation, i.e., we could find no IgA^+ Id^+ plasma cells and no serum IgA paraprotein.

These results contrast with the extent of clonal involvement in another patient with a serum IgM paraprotein that had binding specificity for intermediate filaments (IMF). The involved clone also included IgG_1, IgG_3, IgA_1, and IgA_2 plasma cells which were identified by the homologous idiotype, V_H subgroup, and antigen specificity (A. Landay, H. Kubagawa, and M. D. Cooper, unpublished).

It is puzzling that different members of a malignant B clone can behave so differently with regard to proliferation and differentiation. It is of course possible that, like normal B cells, they are influenced differently by antigens and immunoregulatory T cells (e.g., see chapter by Gershon). The problem is usually complicated by the unknown antibody specificity of the immunoglobulins made by malignant B cells. In the above example, however, the antibodies were directed against a highly conserved determinant present on all IMF forms. Since it is on a basic cellular constituent, this antigen would be released with cell injury and hence available to stimulate immunocempetent cells. This could explain why individuals with hepatitis often produce high titers of antibodies

to IMF [70]. The mere fact that 5%–10% of the IgM paraproteins in humans have IMF specificity [71] may in itself imply a role for antigens in the malignant behaviour of transformed B-cell clones.

E. Conclusions

These results are consistent with the idea that while B-cell malignancies show great variability in their progression along normal differentiation pathways, they undergo in common an initial transformation process within the bone marrow environment (Fig. 3). An important corollary of this hypothesis is that the events involved in initiation of normal differentiation would also be engaged in the genesis of malignant B-cell clones.

Our data further suggest that the initial transformation process is not always immediately followed by exaggerated overgrowth of the B cells belonging to the affected clone. Lane and her co-workers have obtained evidence in DNA transfection studies suggesting that different transforming genes may be activated in neoplasms featuring pre-B, B, or plasma cells [72]. Their results indicate that specific transforming genes are activated in neoplasms corresponding to specific stages of differentiation within the cell lineage. The hypothesis that human B-cell malignancies involve the sequential activation of at least two transforming genes, as may be the case in the avian lymphoma model [11], would easily accomodate both sets of observations.

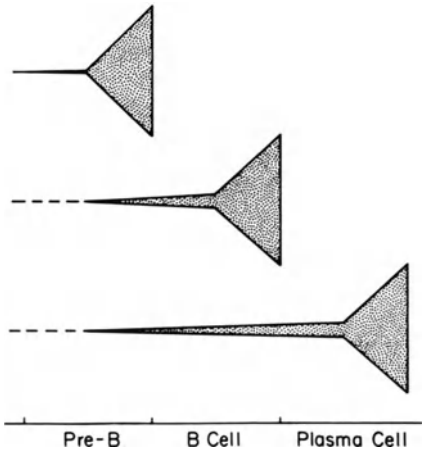

Fig. 3. Extent of involvement and clonal expansion in pre-B, B, and plasma cell malignancies

Still to be explained is the great variability in the growth and differentiation behavior of different members within neoplastic B-cell clones, and why B-cell clones with certain antigen specificities are more frequently involved. In view of these features, and the demonstration that immunoregulatory T cells can modify growth and differentiation of neoplastic B-cell clones [73, 74], it is still plausible that antigens and T cells may be significant modifiers of human B-cell tumors. It should be mentioned, however, that pre-B cell leukemias represent a clear exception to the idea that antigens may influence growth and differentiation of neoplastic B cells, and since the pre-B leukemia cells lack the surface immunoglobulin with which to see antigen, they would not be expected to be clonally regulated by the usual immunoregulatory controls.

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Transfection as an Approach to Understanding Membrane Glycoproteins*

A. Fortunato, R. F. L. James, A. Mellor, and N. A. Mitchison

Gene transfection has much to contribute to our understanding of membrane glycoproteins. The technique is in principle simple: it consists of the transfer of a single gene from one cell to another, using the method of DNA recombination in plasmids to manipulate the gene during the transfer and to rescue it for analysis afterwards. This is valuable for several reasons. The first and simplest is that it generates a cell which has a single new gene product. As the functions of most gene products are still unknown, this should greatly help us to find out what these functions are. For example, the function of the great majority of membrane glycoproteins such as Thy 1, Lyt 1, and T5/T8 remains to be understood. Most membrane glycoproteins have so far been defined only as antigens, sometimes and to an increasing extent through the use of monoclonal antibodies. It turns out to be very difficult to find out what these glycoproteins do, even after quite a lot has been found out about their structure. The Thy 1 molecule is a case in point. It was discovered 18 years ago, it has been used as a marker in lymphocyte differentiation for 13 years, and its primary structure has now been unravelled [23], yet we still know next to nothing about its function. Up to a point the classical approaches of genetics can be applied to these problems: analysis by means of loss and temperature-sensitive mutations. Nowadays these may be supplemented by segregation analysis, in which a cell positive for a given glycoprotein and a given function is fused with a negative cell and the daughter cells analysed for co-expression of the glycoprotein and the function [3]. But progress using classical genetics has been slow, and the contribution to be exprected from transfection is accordingly great. With some justice one could argue that transfection is not a new departure in principle since it merely uses positive variants where classical genetics uses negative variants. However there are many reasons for expecting these positive variants to be far more valuable.

While it is the natural functions of membrane glycoproteins which are likely to interest us most, there are other functions – sometimes termed "pseudofunctions" – which are well worth attention. Activity as an antigen is a good example. Mammalian membrane glycoproteins generally function as antigens only in the course of clinical procedures such as blood transfusion and organ transplantation, or in experiments. Yet this activity is of great interest, not only because of its importance in influencing the outcome of clinical procedures, but also because of the information which allo-immunization provides about the working of the immune system [14]. This is one of the areas in which transfection is likely to have a major impact.

Apart from its application in understanding the function and pseudofunction of gene products, transfection is of importance in formal and reversed genetics. In formal genetics transfection can be used as a starting point for examining gene structure, and for locating and enumerating genes within the genome. Its use for these purposes is

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complementary and to some extent competitive with biochemical approaches to gene cloning. It provides a method of constructing DNA probes which is alternative to the standard route of isolating specific mRNA and proceeding via cDNA. In reversed genetics – still largely an undeveloped subject – it is envisaged that genes will be varied structurally prior to transfection. This will generate altered products of structural genes, primarily for use in structure-function analysis. It will also generate genes with altered control sequences, for use in analysis of the control of transcription.

As a technique for analysing the function of glycoproteins transfection does not stand alone. Perhaps its most formidable competitor is the use of liposomes. Individual glycoproteins can be isolated by means of standard methods in protein chemistry, and then incorporated into liposomes for functional testing. This approach is being followed, for example, in studies of cytotoxicity mediated by T cells [8]. It is yielding results of interest, and there is scope for considerable extension of the approach if and when genetically engineered glycoproteins can be produced in bulk. At present it has the disadvantage that only very limited quantities of the glycoproteins in interest can be produced, and there are problems about purity.

A. Transfection in Immunology

We present a list of the future applications of transfection in cellular immunology which seem to us particularly urgent. Obviously any such list is arbitrary and incomplete, and reflects our individual interests. Nevertheless we hope that it may be useful in provoking further thought by ourselves and others.

I. Unification of Function

Cell surface markers have often attracted interest from several points of view, and have accordingly been studied by means of several different assays. In such cases there may be doubt whether one is dealing with one or several distinct membrane molecules. A case in point is the major histocompatibility complex, where products of the class II region have been studied as antibody-defined alloantigens, as lymphocyte-defined alloantigens, as "restriction elements" or guides for self-lymphocytes, and as controllers of immune responsiveness. Another is H-Y, which has been studied as an alloantigen defined by cytotoxic T cells, helper T cells, suppressor T cells and antibodies, and also as a controller of sexual differentiation [22]. In the case of the MHC it is argued that all these functions are exercised by each of the members of a small family of glycoproteins, which probably number no more than two for the class II molecules of the mouse [9]. This view, the "unified" view of the MHC, rests on evidence from two sources: analysis of spontaneously occurring mutations and the use of monoclonal antibodies. It is likely that transfection experiments will further document this unification, and indeed this process has already begun [12]. It will be more interesting to see what happens in the case of H-Y, where the outcome is more in question.

II. Alloantigens: The Ultimate Congenic Lines

In the past alloantigens have been defined and studied through the use of congenic strains and recombinants between congenic strains. Most of this work has been done in the mouse, where the production of congenic strains and their recombinants has become a major and very expensive international industry. This work is being duplicated in other even more expensive species, such as the rat and the chicken. In the chicken, because of the shorter genetic length of the MHC, ten times as many animals will be needed in each informative cross [18] (unfortunately cell lines are hard to produce in chicken, and no transfection-susceptible cell is yet available). At the end of all this effort is still uncertainty about functions defined in this way, mainly because of possible contamination of the desired gene by flanking genetic material. In a recent study, for example, Dresser used an immunoglobulin allotype which had been backcrossed for 20 generations only to find that his results could be attributed to

genetic contamination. This he was able elegantly to identify as a minor transplantation alloantigen [7].

In our own work on alloantigens recognized by regulatory T cells the problem of genetic contamination has been a recurring theme. Thus some years ago we defined a population of helper T cells which appeared to recognize the class I MHC molecular H-2K, but we could not exclude the possibility that their true target was an unidentified class II molecule [17]. More recently we encountered the same kind of problem with a helper cell line recognizing an unexpected H-2A specificity [24]. Looking outside the MHC, we have had problems with Thy 1. On the whole the evidence suggests that T cells do not recognize Thy 1, and in a primary response system colleagues in this laboratory have shown that helper T cells definitely do not do so [11]. Nevertheless hyperimmunization appears to generate a population of helper cells which do recognize the molecule, if we assume that our Thy 1 congenic strains are truly congenic [4]. Obviously this assumption is open to question.

III. Transfection-Generated Congenics and the Genetics of Immunological Responsiveness

The genetic control of immunological responsiveness to alloantigens has become an active subject. Several careful studies of responsiveness to MHC molecules are under way elsewhere [5] and colleagues in this laboratory and elsewhere have tried to analyse responsiveness to Thy 1 [6, 25]. The work is going slowly because it is limited by the availability of congenic strains. There are few instances where an alloantigen is available on a medium range of paired congenic strains, and none except H-Y where such pairs are really widely distributed. That is one reason why the genetics of responsiveness to H-Y has attracted so much interest [19, 21], and why it has led to fundamental advances in our understanding of this whole subject (for an example of this see our account of the work of M. Brenan [15]). Transfection could greatly broaden the material suited to this kind of analysis.

IV. Characterization of Minor Alloantigens

H-Y has already been mentioned as an alloantigen defined by T cells, and it was in fact discovered in this way. The same is true of most other minor alloantigens, and indeed of some medial alloantigens as well. Nearly all these minor antigens can at present be defined only by means of T-cell responses [1]. The same applies to tumour-specific transplantation antigens, including not only the classical antigens of chemically induced tumours in mice, but also the very interesting antigens defined recently on UV-induced tumours [10] and mutagenized "tum" variants [2]. These UV tumour antigens are arguably the counterpart in mice of the only antigens known to mediate immune surveillance in man [16]. We have long been interested in murine minor alloantigens because of the range of reactivity with regulatory T cells which they offer. One may rationally hope that this range of reactivity will eventually generate rules about how to construct a helper or suppressor epitope [13]. But we cannot properly exploit all the immunological information without having also the relevant structural information. And for T-cell defined antigens this information has long seemed inaccessible. All this has now been changed by the advent of transfection. In fact we can now expect to have DNA sequence information about minor antigens long before we have protein chemistry.

V.

We close this list simply by mentioning two molecules central to modern immunology, IgD and I-J, one of which has a structure without a function and the other a function without a structure. Both of these enigmas we believe will be resolved by means of transfection.

B. Recent Work on Transfection

We, in collaboration with colleagues elsewhere, have been transfecting mouse genes encoding membrane glycoproteins belonging in class I of the MHC [12]. Several groups have already isolated cDNA and

genomic DNA clones containing class I MHC genes of the mouse (cited in [12]). In order to screen our cosmid library we used cDNA probes obtained from elsewhere. We did in fact carry out some initial work on screening directly by expression, and we believe that in the long run this may prove a viable strategy for screening genomic clones for other glycoproteins such as Thy 1. Detailed information about the preparation and screening of the cosmids is presented elsewhere in this volume by E. Weiss, and we present here further characterization of the transfected cells. In the initial report we described a radioimmunoassay which detected expression of transfected H-2K^b and H-2D^b genes by means of monoclonal antibodies. We here present fluorescence-activated cell sorter analysis of these transfected cells using the same monoclonals.

Table 1. Control titration of anti-H-2K^b (Y25) and anti-H-2D^b (B22.249.RI) monoclonal antibodies on L cells (C3H) and EL4 cells (B10)

Antibody titration	Cells used[a]	
	EL4	LDI
NMS 25[b]	440± 126	521± 63
Y25 (anti-K^b)		
2×10^{-3}	8,650± 917	637±123
6×10^{-3}	7,620± 805	641± 49
18×10^{-3}	6,848±1,246	NT
54×10^{-3}	3,461± 834	NT
162×10^{-3}	1,344± 412	NT
B22 (anti-D^b)		
40	15,355± 353	574±129
120	7,736± 335	483± 67
360	2,776± 158	NT
1,080	1,080± 52	NT
3,240	595± 16	NT

The monoclonal antibodies used for screening transfected LD1 cells were titrated by a radiobinding assay utilizing affinity-purified ^{125}I-rabbit anti-MIg as the second label. EL4 cells (H-2^b) were used as the positive control. Antibodies used were Y25 (anti-H-2K^b) (kindly provided by Dr. E. A. Lerner and B22.249 RI (anti-H-2D^b) (kindly provided by Dr. L. Herzenberg)

[a] Results expressed as ^{125}-I-rabbit anti-MIg c.p.m. bound ± S.D. (total counts applied = 100,000)

[b] Titration expressed as the reciprocal of the antiserum dilution used

Table 2. Fluorescent activated cell sorter analysis of mouse L cells (H-2^k) transfected with H-2K^b and H-2D^b genes

Cells	Antisera			
	NMS 1/100	Y25 (αK^b) 1/1000	B22 (αD^b) 1/100	αH-2^k 1/100
LDI	7	7	7	99
EL4	7	96	79	13
LH8	8	40	7	NT
LH8.1	6	94	11	98
LH8.2	16	98	NT	NT
LB3.2G	16	9	54	100
LB1.1.1	7	NT	50	95

Results are expressed as the percent positive cells over background level (cells plus FITC-anti-MIg). FACS analysis of transfected LD1 cells are expressed as the percent positive cells (gates 41 – 255) over the background level (set at gates 1 – 40). Antisera used: NMS 1/100, normal mouse serum; Y25 1/1000, monoclonal anti-H-2K^b); B22 1/100, monoclonal anti-H-2D^b; B10 αC3H, 1/100, allo-antiserum (absorbed EL4 cells). Cells used: LD1, Tk$^-$ mouse L cells (H- 2^k); EL4, mouse T-cell lymphoma (H-2^b); LH8, LD1 cells transformed with H-2K^b gene (uncloned); LH8.1/LH8.2, LD1 cells transformed with H-2K^b gene (cloned); LB3.2G/LB1.1.1, LD1 cells transformed with H-2D^b gene (uncloned)

Control data showing the expression of K^b and D^b on B10 (H-2^b) cells (EL4) and the lack of expression on C3H (H-2^k) cells (L) are presented in Table 1. The same monoclonals were then employed for FACS analysis shown in Fig. 1. In this analysis the cloned cell line LH 8.1 (H-2^k transfected with K^b) shows as essentially 100% K^{b+} and 0% D^{b+}, while the as yet cell line LB3.2G (H-2^k transfected with D^b) shows as essentially 0% K^{b+}, and with a major fraction of cells D^{b+}. The corresponding numerical data from this FACS analysis are presented in Table 2.

What these data establish is that transfected gene products can successfully be picked up on FACS analysis. This is a step forward towards screening by expression,

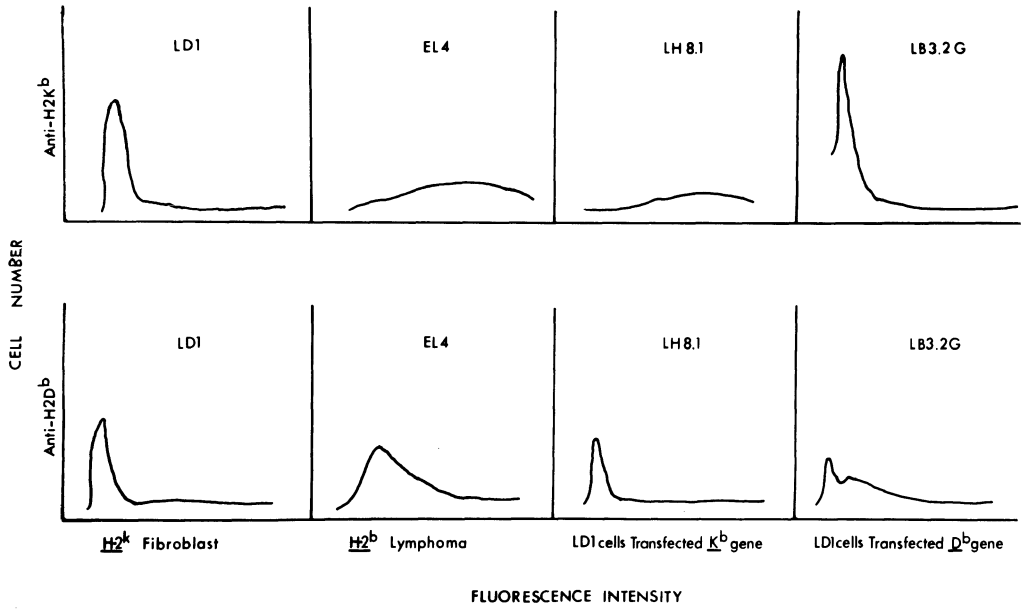

Fig. 1. Fluorescence-activated cell sorting (FACS) using the Beckton-Dickinson FACS IV. For each experiment the laser power was set at 200 mW, the photomultiplier at 730 V, the scatter gain at 4/0.8 and the fluorescent gain at 16/0.8. Generally cells falling between gates 1–40 were considered negative while those falling between gates 41–255 were positive. A total of 10^4 cells were considered in each profile. Cells used: LD1, Tk^- mouse L cells (H-2^k); EL4, mouse T-cell lymphoma (H-2^b); LH 8.1, LD1 cells transformed with H-2K^b gene (cloned line); LB 3.2G, LD1 cells transformed with H-2D^b gene (uncloned line). Monoclonal antibodies used: Y25, anti-H-2K^b (1/1000); B22.249.RI, anti-H-2D^b (1/100)

where it is expected that FACS analysis will be used to clone cells positive for expression.

C. Further Prospects

The recipient cells used in this work are L cells, which unfortunately have lost Thy 1 expression. Using a sensitive immunization assay we have confirmed a previous report to this effect based on immunofluorescence [20]. Our immediate aim is to study regulatory T cells directed at the products of transfected genes, and for this purpose it is almost essential to use Thy 1 as a read out antigen detected by B cells. Accordingly, we are now engaged in transfecting rat cells known to be positive for Thy 1.1. We are also trying to isolate genomic clones encoding Thy 1; it will not have escaped general attention that the published amino acid sequence contains at least a portion attractive for its low ambiguity [23]. In the future the whole programme outlined in our introductory section lies open.

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Structural and Functional Aspects of the T-Cell Differentiation Antigens T3, T6, and T8

C. Terhorst, J. Borst, P. Lerch, M. van de Rijn, P. Snow, H. Spits, and J. de Vries

A. Introduction

The progressive diversification of T-lymphocytes begins within the thymus gland. Since cell-cell interactions may play a major role in this process, the study of the expression of thymic surface markers would aid our understanding of thymic differentiation. On murine thymocytes, selective expression of genes coding for cell surface markers has been studied with alloantisera [1]. More recently, monoclonal antibodies have made possible the study of selective expression of cell surface glycoproteins on human thymocytes. Thus, as in the mouse, early and late events in the thymic differentiation have been recognized [2]. Although the precise function of these thymic differentiation antigens remains to be determined, it seems plausible that they may govern associative recognition among cooperative cells sets. One could therefore surmise that inappropriate expression of some of the thymic differentiation antigens may play a role in leukemogensis.

The majority of human thymocytes (75%–80%) express the markers T4, T6, and T8, whereas only the most mature thymocytes (15%) can be decorated with the monoclonal reagent anti-T3 tagged with fluorescein-isothiocyanate [2]. T6 has never been found on peripheral blood T cells, whereas T3 is present on all T-lymphocytes. T4 and T8 are present on different subsets of T cells and on $T3^+$ $T6^-$ thymocytes [2]. As the thymocyte is exported into the peripheral T-cell compartment, it is again involved in a variety of cell-cell interactions. Interestingly, the T-cell markers T3 and T8 appear to serve functions which are specific for interactions of the cell type which expresses them.

Here we discuss the preliminary structural analysis of T3, T6, and T8 and the possible role of T3 and T8 in the recognition of target cells by cytotoxic T-lymphocytes. Studies involving the glycoprotein structure, biosynthesis, and membrane insertion of T3, T6, and T8 will, in our opinion, facilitate investigations that develop our understanding of the differentiative pathways of human thymus-derived lymphocytes; aid in the molecular description of T-cell functions; and make further classification of T-cell leukemias possible.

B. The Thymic Differentiation Antigen T6

The T6 antigen is a marker which is found primarily on human thymocytes and thymus-derived leukemias [2, 3]. The only nonthymic cells which have shown positive reactivity with the anti-T6 monoclonals [OKT6(2), NA1/34(3), and IIC7(7)] are Langerhans cells in the skin and in lymph nodes [4].

SDS-polyacrylamide gel electrophoresis revealed that the T6 antigen is a protein with an apparent molecular weight of 49K under both reducing and nonreducing conditions [5]. If the thymocytes were radioactively labeled with tritiated sodium borohydride after mild treatment with 1 m*M* sodium periodate, a similar band of 49K daltons was observed after SDS-polyacrylamide gel electrophoresis of the immunoprecipitate, indicating that the target antigen for anti-T6 is a glycoprotein [5]. A

smaller protein of 12K daltons was detected in experiments starting with ^{125}I-labeled lysates. This protein was not seen after tritium labeling and was subsequently identified as β2 microglobulin [5–7]. Both in mice and humans, β2 microglobulin is a protein found to be associated with class I products of the major histocompatibility complex. Based on tissue distribution and molecular weights, T6 may be considered the human homologue of the mouse TL (thymus leukemia antigens). Using isoelectric focusing as an analysis of ^{125}I-labeled T6 antigens from over 20 thymus preparations and four T leukemic cell lines, we detected no charge heterogeneity between T6 antigens from different individuals. With the exception of T6 from the leukemic cell line, MOLT-4 (15 bands) T6 from all individuals was found in twelve bands [6]. However, upon treatment with neuraminidase, this pattern was reduced to four bands in all cases. As the MOLT-4 T6 contained more sialic acids, and since the MOLT-4 T6 contained forms with a higher molecular weight, we suspected that MOLT-4 T6 could have an extra oligosaccharide sidechain [6].

Further studies using the enzyme endoglycosidase-F, which was found to remove most complex N-linked oligosaccharide chains [7], showed quite surprinsingly that the protein backbone of T6 was 34K. This was supported by deglycosilation experiments using acid hydrolysis with trifluoromethane sulfonic acid, which also resulted in a 34K product [7]. The unglycosilated form of T6 MOLT-4 was also found to be 34K, which suggestes strongly that the MOLT-4 T6 may contain an extra oligosaccharide sidechain. Whether this means that MOLT-4 T6 is an "alien histocompatibility antigen" will need protein sequence analysis.

The difference in glycosylation between HLA-A/B (10%) and T6 (30%–35%) could explain why biosynthetic labeling of T6 was found to be far more difficult than in the case of HLA-A/B antigens [7]. Perhaps, the posttranslational modifications are a rate-limiting step in this process. Quite surprisingly, the expression of β2m-associated proteins, but not T6, can be enhanced by interferon-containing lymphocyte-conditioned medium [7]. Whether this is a reflection of the role of T6 in the thymocyte differentiation process remains to be determined. More importantly, as in the case of TL antigens [8], only a restricted group of thymocytes in the appropriate differentiation stage may be able to express the T6 gene.

The function of the T6 antigen is unknown. It may be a receptor for a thymic hormone or this structure may govern associative interactions between thymocytes. It is of interest to note that T6$^-$ thymocytes strongly express HLA-A/B antigens, whereas T6$^+$ thymocytes stain only weakly with fluoresceinated anti-HLA-A/B reagents [2]. Perhaps these two class I M.H.C. antigens may fulfill a similar function in distinct stages of differentiation of the thymus. Knowledge of the structure and function of T6 will therefore undoubtedly aid in our understanding of thymic differentiation and of leukemogenesis.

C. Complexity of the T-Lymphocyte Cell Surface Antigen T3

The human cell surface marker, recognized by the monoclonal reagent anti-T3 (OKT3 or anti-Leu-4), was found most strongly on mature thymocytes. This is exemplified by the finding that only T3-positive thymocytes were responders in a mixed lymphocyte reaction [2, 9]. All human T-lymphocytes were reactive with the monoclonal antibody anti-T3. Thus far, only cells from the human thymus lineage have been found to be reactive with this monoclonal reagent [2].

The target antigen for anti-T3 appears to be involved in most of the proliferative functions of human T-lymphocytes. Interestingly, anti-T3, and to a much lesser extent the monovalent *Fab* fragment, induced DNA synthesis in human peripheral blood T-lymphocytes. Maximal mitogenesis occurred at concentrations of about 10^{-12} *M* (10). When added in higher concentrations of about 10^{-8} *M*, the antibody (and its *Fab* fragment) blocked proliferative responses to soluble and cell surface antigens, inhibited the generation of cytotoxic T-lymphocytes in a mixed lymphocyte culture, and abrogated the ability of T cells to help B cells in antibody production

[11]. These studies suggest that the T3 antigen is involved in cell-cell interaction and/or receptor functions.

Several glycoproteins were found in the immunoprecipitates prepared with a monoclonal reagent anti-T3 [12]. In addition to the major glycoprotein complex of 20K, glycoproteins of 25–28K, 37K, and 44K were detected in immunoprecipitates. The charge heterogeneity of the 20K and 25–28K proteins was caused by variable sialic acid content. The 20K T3 glycoprotein carried several "complex-type" and endoglycosidase-H sensitive sugar moieties. We found that the 20K protein could be labeled with ^{125}I-iodonapthylazide, a reagent that reacts with hydrophobic areas of proteins [12]. Charge shift electrophoresis experiments showed that the 20K, 25–28K, 37K, and 44K glycoproteins are associated in a detergent-soluble complex [14].

Biosynthetic studies and experiments using endoglycosidases demonstrated that:

1. The nonglycosylated precursor of 20K T3 is 14K [13].
2. The nonglycosylated precursor of the 25–28K protein is 16K [13].
3. A nonglycosylated 20K T3 exists which is labeled strongly with ^{125}I-iodonaphthylazide.

Analysis by two-dimensional gel electrophoresis provided evidence that the 20K and 25–28K T3 from resting T-lymphocytes, PHA-activated T-lymphocytes, CTL clones, and a T leukemic cell line HPB-ALL are virtually identical [11, 20].

D. Structure of the T-cell Subset Antigen T8

The target structures recognized by the monoclonal antibodies anti-T5 (OKT5), anti-T8 (OKT-8), or anti-Leu-2A on human T-lymphocytes are borne by a dimer of two sulfhydryl-bridged 33K glycoproteins. Upon reduction and alkylation of NP-40 solubilized membrane proteins, only OKT-8 precipitates the 33K monomer, whereas the target structures of OKT5 and anti-Leu-2A are probably borne by the dimeric structure only. In SDS gels, the dimer migrates either as a 67K protein or a 76K protein. As no other component was found upon reduction than the 33K glycoprotein, and since peptide maps of the 76K form were identical to those of the 67K form, we assume that this phenomenon is caused by differences in protein folding [15, 16].

Peptide mapping and two dimensional gel electrophoresis detected no structural differences between the target antigens for OKT-5, OKT-8, and anti-Leu-2A on T cells or thymocytes. For reasons of simplicity this glycoprotein will be termed T8, throughout this paper.

On thymocytes, the 33K is associated with a 45K glycoprotein via sulfhydryl bridges. Upon SDS-gel electrophoresis under nonreducing conditions, multimers of 140K, 150K, 200K, 220K, and 260/280K were found in addition to the 67K and 76K dimers [17]. When T8 was precipitated from detergent-solubilized thymocyte membrane protein preparations after reduction and alkylation, only the 33K band was found. This indicated that the T8 determinant was expressed on the 33K glycoprotein only. Whether T8 is associated with the 45K protein on all thymocytes or on $T6^+$ thymocytes is currently being investigated.

Attempts to remove glycan sidechains of T8 with endoglycosidase-H and endoglycosidase-F were unsuccessful [16]. After treatment with trifluoromethane sulfonic acid, the molecular weight of the 33K glycoprotein was reduced to 30K. This would allow for one *N*-linked oligosaccharide or several *O*-linked glycan sidechains. However, this 30K form may still contain carbohydrate [16], and the molecular weight of the T8 polypeptide chain is therefore unknown.

Studies using the hydrophobic-labeling reagent ^{125}I-iodonaphthylazide demonstrated that T8 contains a hydrophobic pocket. This suggested that T8 is an integral membrane glycoprotein.

Taken together, the tissue distribution of T8 [2] and the preliminary biochemical data suggest thatT8 is a human homologue of the murine Lyt 2, 3 antigens. The finding that T8 (see below) and Lyt 2, 3 antigens [18] are involved in the target recognition by cytotoxic T-lymphocytes supports that idea.

E. The Role of T3 and T8 in Antigen-Specific Cytotoxicity Mediated By $T8^+$ and $T4^+$ Human Cytotoxic T-Cell Clones

The nature of the antigen recognition structure(s) on human cytotoxic thymus-derived T-lymphocytes (CTL) is still an unresolved issue. The availability of clones of human CTL will facilitate the analysis of individual CTL target cell interactions on a functional and molecular level. We previously demonstrated that stable CTL clones could be derived from tertiary MLC in which cells of an Epstein Barr virus transformed B-cell line (JY) were used as stimulator cells [19, 20]. More recently, four such clones, which have different antigenic specificities and phenotypes, were used to investigate the role of T-cell antigens T3 and T8 in effector/target cell interaction [20, 21]. Four clones are described in Table 1 which differ in phenotype and specificity. The T8-positive clones HG-31 and JR-2-16 were shown to react only with target cells carrying the serologically defined HLA B7 and HLA A2 determinants respectively, confirming the results of a recent study in which these clones were tested on a large panel of HLA-typed lymphocytes [22]. The T4-positive CTL clone HG-38 was strongly cytotoxic for JY cells only. The T4-positive CTL clone JR-2-19 was cytotoxic for cells expressing HLA DR-2 and HLA DR-6. This reactivity pattern cannot be attributed to a mixture of a HLA DR-2 specific and a HLA DR-6 specific clone since subclones of JR-2-19 reacted in a similar way. It may be possible that clone JR-2-19 recognizes a determinant shared by the DR-2 and DR-6 antigens. Alternatively, this clone may recognize an antigenic determinant on a molecule which is distinct from DR-2 and DR-6, like the recently described DC1 specificity [23] that has been shown to be a molecular complex dinstinct from DR-1, -2, and -6, but which is in linkage disequilibrium with these specificities.

Two monoclonal reagents, OKT-8 and anti-Leu-2A, inhibited the cytotoxic reactions of the $T8^+$ clones HG-31 and JR-2-16 [20, 21], whereas these antibodies did not affect the cytotoxic reactions mediated by the $T4^+$ clones HG-38 and JR-2-19. Anti-T3 antibodies were found to inhibit the cytotoxic reactivity of all four CTL clones. In contrast, OKT-1 and OKT-4 had no effect on the lytic activity of the CTL clones. The inhibitory effect of the monoclonal antibodies anti-T3 and anti-T8 could not be attributed to a functional inactivation of the CTL clones, since ConA overcame the inhibition of cytotoxicity without affecting the binding of the monoclonal antibodies to the clones. These findings indicate that the monoclonal antibodies anti-T3 and an-

Table 1. The cytotoxic activity of the CTL clones JR-2-16, JR-2-19, HG-31, and HG-38 against 12 EBV transformed lymphoblastoid B-cell line cells and one Burkitt lymphoma cell line (Daudi)

Target cell	HLA phenotype				% specific lysis CTL clone			
	A	B	C	DR	JR-2-16	JR-2-19	HG-31	HG-38
JY	2	7		4, 6	80.0	49.8	47.4	46.2
Daudi	–	–		6	2.2	42.8	4.4	10.1
HHK	3	7		6	10.6	46.4	48.3	6.4
EKR	3	7		7	15.9	−1.5	42.2	−0.4
NOB	3	7		2	2.3	69.9	62.6	0.1
PHS	3	7		2	11.3	68.2	52.2	−2.8
SB	1, 2	17, w44		2, 3	79.3	46.7	14.3	10.1
HAR	1	8		7	−0.2	2.2	3.7	2.1
MvL	w32	27	w2	1	−1.7	3.9	2.0	3.2
APD	1	40		6	NT	54.8	7.0	16.1
CJO	11	w35	w4	1	1.7	−0.8	−1.8	−2.6
OOS	26	w22	w2	1	NT	3.8	8.5	5.7
WT	2, 11	27, 55		1	82.5	2.8	0	−0.1

CTL clone	Trypsin treatment	% specific lysis			
		Directly after trypsin treatment		After 24 h incubation at 37° C	
		2.5[a]	5	2.5	5
JR-2-16	Yes	3.9	15.2	72.0	100.1
	No	39.3	81.8	56.0	88.0
JR-2-19	Yes	42.3	50.9	41.5	60.8
	No	54.8	59.8	57.5	69.7
HG-31	Yes	2.9	6.1	59.7	85.9
	No	42.0	56.0	55.7	89.4
HG-38	Yes	13.3	32.7	13.4	41.8
	No	18.4	45.3	15.4	50.1

[a] Effector/target cell ratio

Table 2. The cytotoxic activity of the CTL clones on JY cells after trypsin treatment

ti-T8 blocked the recognition process, but not the cytotolytic effector mechanism itself.

In an attempt to study the role of T3 and T8 in the CTL effector function in more detail, a series of experiments was conducted using proteolytic enzymes. An example is shown in Table 2. Trypsinization of CTL clones JR-2-16 and HG-31 during 10 min at 37 °C resulted in a complete abrogation of the cytotoxic activity of these clones, whereas the cytotoxic activity was fully restored after incubation in IL-2 containing medium at 37 °C and 5% CO_2 overnight. In contrast, the cytotoxic activity of clones HG-38 and JR-2-19 was minimally affected by trypsinization. The disappearance of cytotoxicity after trypsin treatment correlated with the removal of the T1 and T8 determinants. However, the expression of T3, T4, HLA/A/B/C heavy chain and HLA DR remained unaltered. Both the expression of T8 and T1 determinants was restored after overnight incubation at 37 °C and 5% CO_2 (Table 2).

More detailed analysis of the effects of trypsin treatment on the cytotoxic activity of the CTL clones revealed that the cytotoxic activity of the clones HG-31 and JR-2-16 was already abolished after a trypsin treatment of 1 min (Table 3). At that time, the OKT-8 and Leu-2A determinants were still present, although the fluorescence intensity as measured with the FACS IV was decreased [21].

Trypsin treatment of the CTL clones during 2.5 min completely removed the Leu-2A and OKT-8 determinants. In contrast, the lectin-dependent cytotoxicity mediated by the clones HG-31 and JR-2-16 was found to be still intact after a trypsin treatment of 30 min (Table 4). This latter observation indicates that the general ability to lyse target cells is not lost by the trypsin treatment, but suggests that trypsin treatment affects the recognition phase of CTL clone target cell interaction in which T8 is involved.

These results show that the recognition unit for class I antigens on the $T8^+$ CTL is more sensitive to trypsin than the receptor(s) for class II antigen on the $T4^+$ clones. Hydrolysis of the T8 glycoprotein is synchronous with the disappearance of the target recognition by the $T8^+$ clones. Apparently trypsin cleaves off the determinant involved in cytotoxicity before it removes the T8 epitope. Whether the T8 antigen interacts directly with the class I antigen on the target cell or via an associated protein remains to be determined. These studies also do not exclude the possibility that the trypsin sensitivity of T8 expression and cytotoxicity are purely coincidental.

The role of the T3 glycoproteins in the cytotoxic function remains unclear. The inhibition of cytotoxicity of CTL clones by OKT3 described here confirmed previous reports in which "bulk" CTL cultures were used, but thus far it has not been reported

Table 3. The expression of cell surface antigens after trypsin treatment of the CTL clones HG-31 and HG-38

Monoclonal antibody	% of the cells reacting with monoclonal antibody					
	HG-31			HG-38		
	Control	Trypsin treated	Trypsin treated and incubated for 24 h	Control	Trypsin treated	Trypsin treated and incubated for 24 h
a HLA A, B, and C (w6/32)	100 (+ +)	100 (+ +)		100 (+ +)	100 (+ +)	
a HLA DR (OKIa 1)	100 (+ +)	100 (+ +)		100 (+ +)	100 (+ +)	
OKT-1	80 (+)	< 1	100 (+ +)	95 (+ +)	< 1	100 (+ +)
OKT-3	100 (+ +)	100 (+ + +)		100 (+ +)	100 (+ + +)	
OKT-4	< 1	< 1		95 (+ +)	95 (+ +)	
OKT-8	100 (+ + +)	< 1	100 (+ +)	< 1	< 1	
Leu 2a	100 (+ + +)	< 1	100 (+ +)	< 1	< 1	
Leu 4	95 (+ +)	100 (+ +)		95 (+ +)	95 (+ +)	

(+), (+ +), or (+ + +) indicate the relative fluorescence intensity

Table 4. The effect of trypsin treatment on the expression of the monoclonal antibodies OKT-8 and Leu 2a and the cytotoxic activity of the CTL clones HG-31 and JR-2-16 against JY in the presence and absence of ConA

Trypsin treatment during	% of cells reacting with		% specific lysis			
	OKT-8	Leu 2a	HG-31		JR-2-16	
			JY	JY + ConA	JY	JY + ConA
0 min	97% (+ + +)	96% (+ + +)	36.8	37.0	54.1	62.3
1 min	81% (+)	82% (+)	1.0	28.7	10.2	59.1
2.5 min	< 1% (−)	< 1% (−)	0.4	40.1	1.8	58.6
5 min	< 1%	< 1%	0.6	41.9	2.0	55.1
10 min	< 1%	< 1%	0.1	39.9	3.1	50.2
30 min	1%	1%	−0.2	36.4	2.0	53.4

(−), (+), or (+ + +) indicate the relative fluorescence intensity

that Leu-4 can also inhibit cytotoxicity. The neutralizing effect of ConA on the inhibition by Leu-4 and OKT3 suggests that this antigen is involved in some stage of the recognition process. But, the trypsin experiments showed that T3 must be involved in a different phase of the target cell recognition than T8 [21]. This has also been reported by other [24]. Chang et al. [25] raised the possibility that the T3 molecular complex is part of the T-cell antigen receptor.

However, several findings argue against this possibility. Firstly, although the cytotoxicity of the clones HG-31 and JR-2-16 was highly susceptible to trypsin treatment, the expression of OKT-3 remained unaltered. Moreover, trypsin treatment did not influence the cytotoxicity of the clones HG-38 and JR-2-19, which indicates that

the antigen recognition structure of clones HG-31 and JR-2-16 is different from that on HG-38 and JR-2-19. Secondly, if the T3 complex itself is the antigen receptor, it would be expected that there is a structural difference in the antigen specificity. However, the two-dimensional electrophoretic patterns of the OKT-3 precipitated material from both clones were identical [21]. Taken together, these findings suggest that the 20K T3 and 25–28K T3 itself may not serve as the T-cell receptor for alloantigens. However, the T-cell receptors could be associated with the T3 structure perhaps through interactions with hydrophobic nonglycosylated 20K T3. Further biochemical studies including cross-linking around T3 and lysostripping experiments with anti-T3 using several different CTL clones derived from one individual and directed at different class I and class II antigens will probably provide evidence for associations between T-cell receptors and the T3 complex.

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Human Histocompatibility Antigens*

H. L. Ploegh

A. Introduction

Numerous chapters in this volume testify to the importance of molecular biology the analysis of problems relating to human cancer. Most recently, it was shown that human (onco)genes carrying single nucleotide substitutions may confer the transformed phenotype rather than encode a presumably normal – albeit unknown – cellular function [1]. With reference to the study of leukemia, recent advances have demonstrated the existence of human leukemia viruses (this volume), and undoubtedly their molecular biology will equally be of great interest for our understanding of these tumors.

It is usually assumed that our first line of defense against tumors is the immune system. In the concept of immune surveillance, cytotoxic T-lymphocytes eliminate malignantly transformed cells. Only when such cells escape the immune system – for whatever reason – may a tumor arise. It has been known for some time that T-lymphocytes in their interactions with target cells, either for cooperative or destructive purposes, are guided by the products of the major histocompatibility complex (MHC) [2]. A thorough understanding of the structure and biologic role of this genetic region should contribute to our knowledge of how "foreign" cells are eliminated – and hence, how tumors may be prevented from establishing themselves.

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B. The Major Histocompatibility Complex

Originally discovered through skin grafting in mice and through the characterization of leukocyte-agglutinating antibodies in man, the major histocompatibility complex (MHC) has become of central importance in immunology. In humans, the MHC is located on the short arm of chromosome 6 and spans a region of approximately 1.5 centimorgans. The MHC encodes several kinds of membrane glycoproteins, most conveniently referred to as class I and class II antigens [3]. Class I antigens consist of an MHC-encoded membrane glycoprotein chain of 44,000–48,000 daltons in association with a β_2-microglobulin, which is not encoded by the MHC [4]. Class II antigens are built up as a two-chain structure as well, but both subunits are anchored to the membrane and – at least in the mouse – both subunits are encoded by the MHC [5].

The single most striking feature of the MHC is the polymorphism of its products. Both in man and in the mouse, some 30–50 different alleles are likely to exist at the different class I loci (*H-2K, D,* and *L* in the mouse, *HLA-A, -B,* and *-C* in man, to name but the most familiar ones) and a similar situation probably applies to class II antigens [6]. The molecular nature of this polymorphism has been the object of intense study, and the picture that emerges points to the importance of amino acid substitutions in the polymorphic chains in generating the different alleles. Studies of mouse H-2 mutants have been particularly informative in this respect [7]. So far, the

possibility that the MHC encodes carbohydrate rather than protein determinants has received attention in passing. The difficulties of structural analysis of complex carbohydrate structures (e.g., glycolipids) is one of the reasons this area has remained relatively underdeveloped.

The polymorphism of the MHC is thought to be of functional significance. The associations with disease in man constitute a clear argument: Certain diseases are found with elevated frequencies in individuals carrying particular alleles or a constitution of alleles (haplotypes) at the *HLA* loci [6]. Quite often these diseases are, or are thought to be, associated with the immune system.

Thus, a thorough analysis of the polymorphism of the MHC and its functional ramifications should be carried out. Peptide-mapping and protein-sequencing studies have been the methods of choice in the past, but more recently recombinant DNA methodology has gained a foothold. At present, this area is undergoing explosive development, and at the time of writing cDNA clones for virtually every class of MHC-encoded protein have been obtained. Using these cDNA clones, large sections of the murine MHC have been cloned, and in the near future we shall see the construction of a complete physical map of the MHC [8, 9].

C. Class I Genes

By classical serology and immunogenetics, three class I loci have been defined in man so far – *HLA-A, -B,* and *-C* [6]. However, analysis of total human genomic DNA by Southern blotting has revealed the presence of at least 20 class-I-related sequences [10]. In the mouse, the same situation is found and has in fact been studied with higher resolution – 36 class I genes have been cloned from the BALB/c genome [11]. The number of class I antigens identified by genetics and serology is far smaller, however [12]. How can we explain this discrepancy?

First, it is possible that a large number of class I genes are expressed only in specialized tissue or cell types, making their detection by immunogenetic and serologic means difficult. Secondly, it is possible that we are dealing with a relatively large number of pseudogenes. Experiments in the murine system suggest that the latter possibility is at least in part correct: A number of class I pseudogenes have been identified [13, 14].

To address this question, we decided to investigate a parameter thought to relate directly to gene activity. It has been demonstrated in a number of systems that the state of methylation of eukaryotic DNA correlates with gene activity [15]. Hypermethylation of the CpG dinucleotide is generally found to be associated with a lack of gene activity, whereas hypomethylation at these sites is found in actively transcribed genes [15]. The state of methylation can be rapidly assessed by using the isoschizomeric pairs of enzymes Hpa II and Msp I, the latter being insensitive to the presence of methyl-cytosine in the CpG dinucleotide, whereas Hpa II is sensitive.

When human DNA was digested with EcoRI, followed by digestion with Msp I or Hpa II, a clear-cut result was obtained (Fig. 1), namely that the vast majority of human class I sequences, as detected by Southern blotting using an HLA cDNA clone as a probe, were hypermethylated. In the light of current opinion, this would imply that such genes are in an inactive state. Provision has to be made, of course, for the fact that no fine mapping can be carried out due to the presence of a large number of homologous sequences. When the pattern of methylation was examined in DNAs obtained from different tissues of the same individual, no differences were apparent. This would suggest that the degree of methylation of human class I sequences, as detected by these methods, does not vary much from one tissue to another. Secondly, it is apparent that few differences in banding patterns are observed between the different individuals. Although we are dealing with a serologically extremely polymorphic system, this polymorphism is not readily apparent at the level of the DNA that encodes these antigens. A larger survey of enzymes, with different recognition sequences, indeed shows that such polymorphisms can be visualized [10, 16], but the efforts involved are too great when compared with the relative ease of serologic

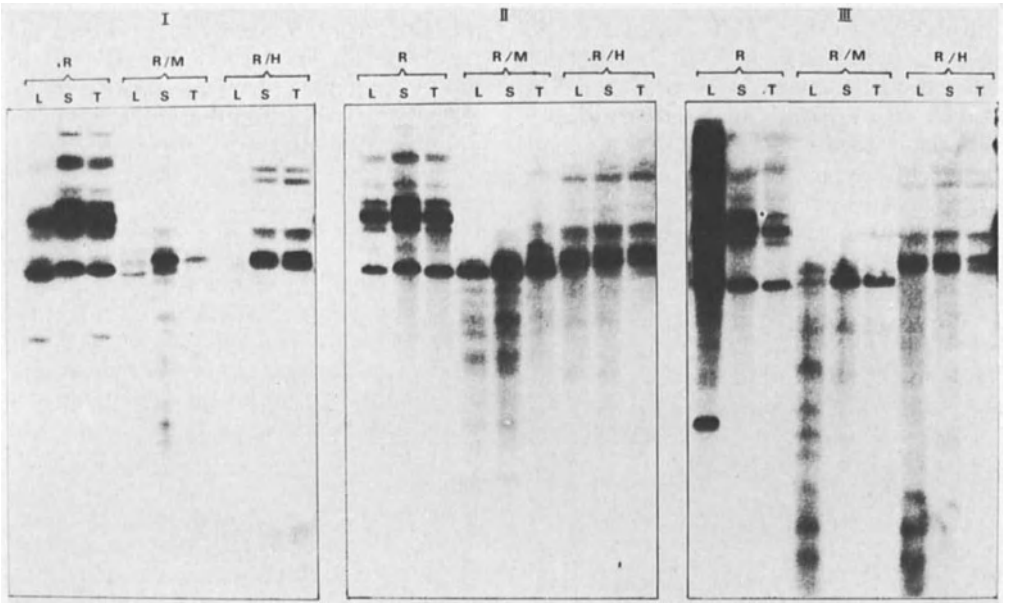

Fig. 1. Analysis by Southern blotting of human class I sequences. DNA from three different individuals was digested with Eco RI, and subsequently with Msp I or Hpa II, as indicated in the *figure*. After agarose gel electrophoresis of the DNA fragments on a 0.8% gel, DNA was transferred to a nitrocellulose filter and probed with nick-translated HLA cDNA clone. Note the apparent lack of polymorphism between the different tissues obtained from a single individual (liver, *L*; spleen, *S*; thymus, *T*) and between different individuals. Note also that most hybridizing sequences are not cut by Hpa II, indicating they are methylated

typing or the resolution of protein-chemical analysis.

D. Class I Antigens

A slightly different approach would be to ask how many gene products can actually be detected. It should be borne in mind that usually alleles at the *HLA* loci are defined by alloantibodies, in which single specifities and their cross-reactive specificities are recognized using the best typing sera available. The advent of monoclonal antibodies has made it possible, however, to obtain xenogeneic antibodies of high titer that react with all HLA specifities examined to date. For example, the mouse monoclonal antibody W6/32 reacts with peripheral blood lymphocytes from humans to a similar extent regardless of the donor's HLA type [16]. Similarly, monoclonal anti-β_2-microglobulin antibodies are available [17] that should react with all class I antigens, as defined by their association with β_2-microglobulin. The use of such antibodies, either alone or in combination, should allow the estimation of the number of class I antigens present in a certain cell type, for example, in mitogenically activated lymphocytes. To examine this question, peripheral blood lymphocytes are labeled with ^{35}S-methionine; class I antigens can then be isolated from detergent extracts prepared from such cells after detergent solubilization. Display of the isolated class I antigens by a separating technique with high resolving power then enables direct counting of the number of different genes expressed [18, 19]. For humans, the lack of inbred strains – one of the few advantages of analyzing the murine system – can be compensated for by the use of cells from homozygous donors, usually the offspring of consanguinous marriages. In such so-called homozygous typing cells, the contributions of a single haplotype can be assessed [18]. At this point, it should be mentioned that all class I sequences that hybridize with HLA cDNA clones can be

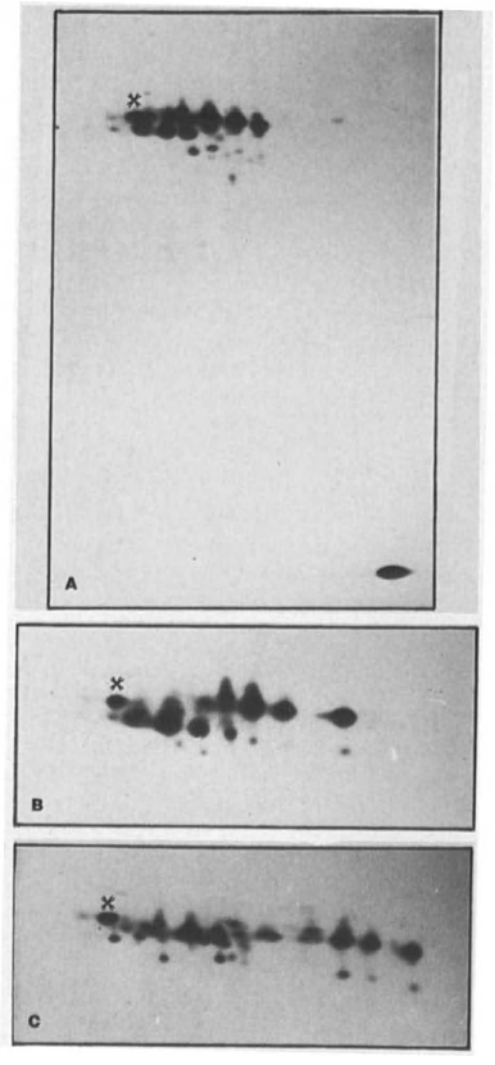

Fig. 2A–C. Analysis of human class I antigens by two-dimensional gel electrophoresis. Class I antigens were isolated by immunoprecipitation with the W6/32 monoclonal anti-HLA antibody and displayed by two-dimensional gel electrophoresis. For detailed methodology see [18]. **A** shows an immunoprecipitate from an HLA-A23, -B7 homozygous individual, the region of the gel from approximately 60,000 daltons to 10,000 daltons being shown. The intense spot at the bottom is β_2-microglobulin. **B** and **C** show immunoprecipitates obtained from HLA-A26, -B56 and HLA-A2, -Bw62 homozygous individuals, respectively. In **B** and **C** only the *HLA* heavy chain region is shown, at a slightly larger magnification than in **A**. The basic end of the gel is oriented toward the *right*, and the position of actin, a common contaminant of such immunoprecipitates, is indicated by *. For further details, see text and [18]

found in man-mouse somatic cell hybrids carrying only the human chromosome 6 [20]. In Fig. 2, the results of such an analysis are given for several different homozygous typing cells. It is apparent that a large number of spots are obtained for each individual donor. This is caused in part by differential sialylation, resulting in the presence of four spots for each specificity. If we take this phenomenon into account, we arrive at a figure of some six class I genes being expressed per haploid genome [18]. Differences in number may be seen according to haplotype, although this matter deserves further investigation. It suffices to say that in the murine system too the complement of class I sequences is thought to show variation according to haplotype. Combined with the results referred to in the previous section, we may conclude that, notwithstanding the presence of a large number of class I sequences per haploid genome, only a small minority are usually expressed in PBLs. Evidence from the analysis of homozygous typing cells suggests that at least 80% of the amount of class I antigen per cell is contributed by the HLA-A, -B antigens [18].

It was already hinted at that this observation may find its cause in the presence of a large number of pseudogenes. Selective pressures to increase the polymorphism of class I antigens may have produced a tendency to duplicate the genes encoding them as a means of increasing variability at the level of the population, as well as that of the individual. A relatively large number of pseudogenes may be the price the organism has to pay for the ability to duplicate given sets of genes. This process may not be altogether harmful nor constitute a great genetic burden since the genes present in the genome, pseudo- or otherwise, are still a substrate for unequal crossing-over events, gene conversions, and the like. Thus the information contained in them can be acted upon by natural selection.

E. Structure of Class I Genes

Using the available cDNA clones as probes, genomic clones have been isolated, and their structure has been determined

([13, 14] and Ploegh et al., in preparation). The structure of a typical class I gene shows an intron-exon organization, which reflects the division of the molecule into what are thought to correspond to functional domains [4]. The signal sequence and the heavy chain domains H1, H2, and H3 are all encoded by separate exons. The membrane-binding region likewise is encoded by a separate exon. A more unusual arrangement is seen in the cytoplasmic part of the molecule, which, in the case of human class I antigens, is interrupted by two introns. It is not clear whether these interruptions imply a functional subdivision at the level of the protein or whether they are fortuitous. It should be kept in mind that for murine class I antigens, highly homologous to their human counterparts, an additional intron is found for the cytoplasmic domain, arguing against a functional subdivision of this part of the molecule as referred to above.

Thus far, little is known about the transcription of *HLA* genes or the factors that exert a regulatory influence. It has been found that interferons increase the expression of HLA antigens by increasing the amount of available mRNA, presumably through action at the transcriptional level [26]. It is of importance to understand these phenomena, since the expression of so-called alien histocompatibility antigens on certain tumors has been postulated being due to the activation of otherwise silent histocompatibility genes. Here, too, a relationship might exist between the ability of the tumor to escape the immune system and the suggestion that "inappropriate" antigens are expressed [27].

F. Variant Antigens

To return to the question of recognition of MHC products by T-lymphocytes, we shall now examine which features on class I antigens may be of importance and can be recognized. As mentioned previously, HLA-A, -B specificities are defined by alloantisera. It was noted by several groups that between HLA-identical individuals cytotoxic T-lymphocytes can be generated that have a specificity for a particular class I antigen, as established by panel and family studies [21, 22]. The conclusion to be drawn from such data is that either variants or subtypes of recognized specificities must exist, or that structures tightly linked to the HLA-A, -B specificities in question can function as targets for cytotoxic T-lymphocytes. A structural analysis of such variant or subtypic antigens has been carried out, and it was found that they were biochemically distinct as well [22, 23]. These initial observations were restricted to two cell lines, one of black and one of oriental racial origin [24]. We carried out similar analyses on a larger panel of white individuals who had been typed for *HLA-A2* by cytotoxic T-lymphocytes and were variant. Our biochemical analysis revealed that these antigens were also biochemically distinct [25]. Peptide mapping and amino-acid sequencing should establish the exact nature of these differences.

These variants or subtypes illustrate the discriminating power of T-lymphocytes recognizing histocompatibility antigens, a feature already well known from the mouse mutant *H-2* work [4, 7]. For subtype identification, the approach outlined in the previous section was used, i.e., isolation of class I antigens by means of a monomorphic monoclonal antibody, followed by separation on a one-dimensional isoelectric focusing gel. This technology affords sufficient resolution to resolve most commonly occurring HLA-A, -B specificities. Although defined by alloantibodies, we can define subtypes without such complex reagents. Such subtypes may not be without clinical significance: If they can be discerned by cytotoxic T-lymphocytes in a cell-mediated lymphocytosis (CML) reaction, it is equally likely that they would be recognized in a clinical transplantation situation. These findings may explain at least in part why matching for HLA-A, -B, -C antigens is of relatively little importance in predicting the outcome of a kidney transplantation. At the same time, techniques such as the present one may identify targets for cytotoxic T-lymphocytes other than those encoded by the *HLA* region. How do these results relate to the problem of human leukemia? Elsewhere in this volume, the reader will find a description of cells transformed with human leukemia virus (HTLV). Such cells express HLA-A, -B, -C

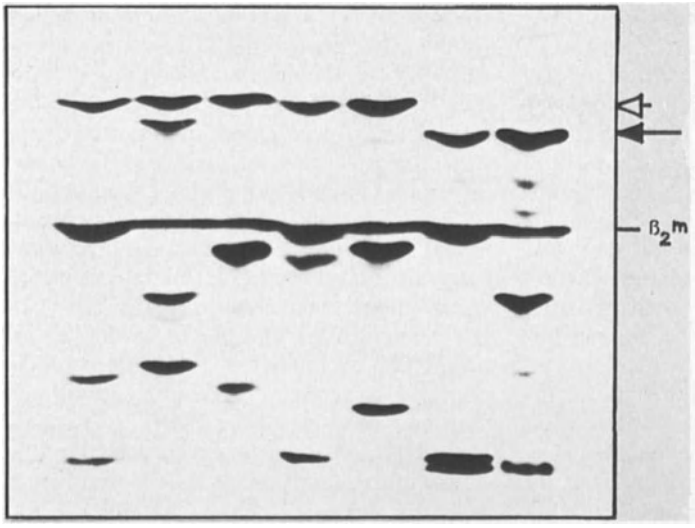

Fig. 3. Analysis of HLA-A2 subtypes by isoelectric focusing. HLA-A, -B, -C antigens were isolated from HLA-A2 positive individuals who could be subdivided according to their reactivity with HLA-A2-specific cytotoxic T-lymphocytes, although indistinguishable serologically. The position of normal A2, as found in 90% of the white human population is indicated by an *arrow*; the position of the minor A2 subtype is indicated by an *open arrow*. The other bands represent other HLA-A, -B specificities present in the different donors. The complexity of such patterns has been reduced by carrying out a neuraminidase digestion on the immunoprecipitates prior to isoelectric focusing. The reader is referred to [19, 22] for further details

antigens other than those found on the original donor lymphocytes. Apparently, there is a link between the transformation event and the expression of HLA antigens at the cell surface. Clearly, a more profound knowledge of how these events interrelate should contribute to our understanding of human leukemia. It would be interesting to analyze biochemically the antigens expressed after transformation by HTLV. Are the HLA-A, -B specificities indeed distinct from those found on the untransformed donor cell, or do they represent unusual post-synthetic modifications? Our description of normal antigens and their subtypes should allow an unequivocal answer to this question.

G. Concluding Remarks

The products of the MHC are amongst the best studied of membrane glycoproteins. We may safely assume that techniques applied to their study will be equally useful for the analysis of other membrane proteins – lymphocyte differentiation antigens and tumor markers. It is equally important to underline once more the central role played by the MHC in the immune response. If the immune surveillance concept is as important as has been assumed so far, and if it is to be understood in detail, the MHC will certainly need further research. There is no shortage of willing hands.

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Structure and Expression of Class I Genes of the Mouse Major Histocompatibility Complex

E. H. Weiss, A. L. Mellor, L. Golden, H. Bud, J. Hurst, R. A. Flavell, E. Simpson, R. James, A. R. M. Townsend, and H. Festenstein

The major histocompatibility complex (MHC) plays an important role in the regulation of the immune response in vertebrates. The MHC in man (*HLA*) and the mouse (*H-2*) is the most thoroughly studied case of a complex of linked genes (see [1] for a review). The *H-2* locus maps on the murine chromosome 17 (Fig. 1). Three different types of proteins are encoded by the *H-2* region. The class I molecules regulate the killing of virus-infected cells. The virus antigen is recognized in association with class I proteins. This is known as MHC restriction of T-cell recognition [2]. The best-characterized class I molecules are the classical transpantation antigens found on virtually all cells, encoded by the *H-2K*, *H-2D*, and *H-2L* loci. Class I antigens are also encoded by the *TL* complex, adjacent to the classical *H-2* loci. The Qa and Tla proteins are lymphoid differentiation antigens. Class I molecules are intrinsic membrane proteins with molecular weights (MW) of 40,000–45,000. They are associated with a smaller polypeptide, β_2 microglobulin, MW 12,000, not encoded by chromosome 17. The class II genes were originally discovered as immune-response genes. They regulate the interaction of T-helper cells and B cells to induce antibody production [3]. The class III molecules encoded by the *H-2* region are complement components. One of the most remarkable properties of the H-2 antigens is their genetic polymorphism. In fact, about 50 alleles at both the *H-2K* and *H-2D* loci have been detected. In this aspect, they differ sharply from almost all other genes. We are particularly interested in how the H-2 polymorphism is generated. We think it likely that the analysis of the class I genes by recombinant DNA techniques will provide some clues to the nature of the polymorphism.

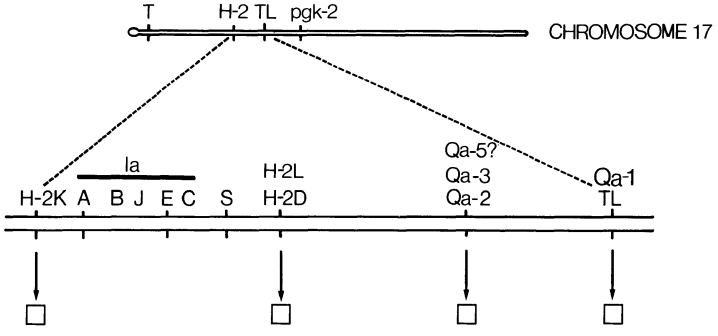

Fig. 1. Genetic map of the murine *H-2* and associated genetic loci. The *top line* shows a diagram of chromosome 17 with the centromere on the *left*. The *bottom line* shows an expanded diagram of the regions between the *H-2K* locus on the *left* and the *TL* locus on the *right*. H-2 class I molecules (□) are expressed from at least four separate genetic loci as shown by the *arrows* below the *line*

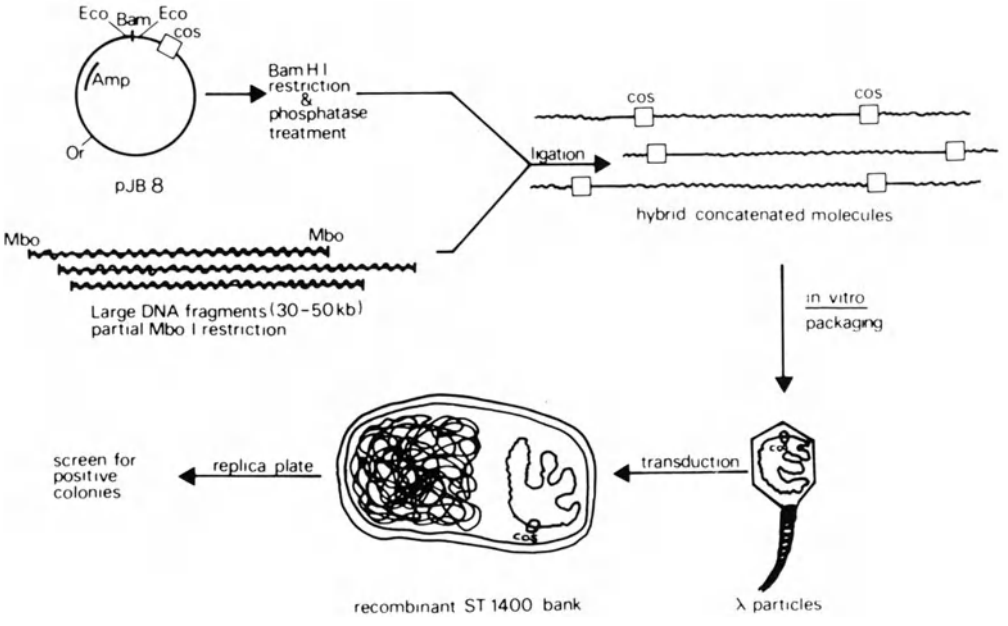

Fig. 2. Scheme for the cosmid cloning techniques

We isolated genes from the mouse major histocompatibility complex by cloning the genome of the B10 mouse (C57/BL10) *H-2*^{b} haplotype, in a cosmid library [4, 5]. The cosmid cloning technique has the advantage that large fragments of DNA (ca. 40,000 bps) are cloned and it is easy to obtain large amounts of recombinant DNA. The principle of the cosmid cloning technique [6] is shown in Fig. 2. Chromosomal spleen DNA was partially digested with the restriction enzyme MboI and the digested DNA size-fractionated on a density gradient. The MboI recognizes a four-bp-restriction site (*GATC*) and cuts the genome frequently. Specific DNA sequences are, therefore, randomly distributed over the gradient. The MboI-restricted fragments can be cloned into the *Bam HI* restriction site (*G GATC C*). Fragments of ca. 40,000 bps are ligated into the Bam site of a vector. A minimal requirement for a cosmid vector is that it contains the cohesive ends of phage λ (cos) and an antibiotic resistance marker for selection in bacteria. The vectors we used to construct the libraries [5] also contained another enzyme gene for selection in eukaryotes. The ligated DNA is packaged in vitro via the cos sequences into λ-particles and transfected into recA$^-$ *Escherichia coli* cells. Once inside the bacterium, the DNA propagates as a plasmid. The complexity of the library was such that the sum of the bacterial colonies covered the murine genome at least four times. Two B10 libraries were constructed, one with the vector pOPF, the other with pTM. pOPF contains the thymidine kinase gene of herpes virus. Cosmids with this vector can be directly transfected into mouse L cells (tk$^-$) and transformants selected in hypoxanthine-aminopterin-thymidine medium. pTM contains the aminoglycoside phosphotransferase gene which makes eukaryotic cells resistant to the drug G418.

We screened the libraries with a human genomic class I gene probe (pHLA 12.4 [7])

Table 1.

Region	No. cosmids	kb	No. genes	Location
1	7	95	2	*H-2K*
2	2	70	1	*H-2D*
3	15	120	5	*Qa2, 3*
4	1	40	1.5	*Qa2, 3*
5	2	50	2	?
6	39	90	5	*TL*

or with mouse H-2 cDNA clones (pH2-III, pH2-IIa [8]). We isolated 82 cosmids containing class I related genes and began a detailed analysis of the structure, sequence organization, and expression of the *H-2*-related genes. By restriction enzyme and southern blotting analysis, the cosmid clones can be grouped into five clusters. We localized each cluster and some single cosmids by polymorphic restriction site mapping to distinct regions on the *H-2* map. The result of these experiments is shown in Table 1. Figure 3 shows in detail how we mapped region *6,* which contains the largest number of cosmids, to the *TL* locus. When a single copy 2.0-kb EcoRI fragment that maps 5′ to the gene *6A* in the region [5] is hybridized to PstI-digested DNA, the fragment detects bands of different size in all three haplotypes (*b, d, k*). AKR DNA ($H\text{-}2^k$) shows a 3.7-kb band, whereas in B10 DNA ($H\text{-}2^b$) the 2.0-kb fragment lights up. The H-2 recombinant mouse strain B6K2 DNA contains a 3.7-kb band and our fragment, therefore, detects the $H\text{-}2^k$ haplotype portion of the recombinant, that is the *TL* locus in B6K2. Region *6* maps to the *TL* locus.

Table 1 shows that from the 82 cosmids containing *H-2*-like genes, two regions map to the loci controlling the expression of the classical transplantation antigens, H-2K and H-2D. The majority of the genes are localized outside the *H-2* locus proper, six genes mapping to the *Qa* locus and five genes to the *TL* locus. It is interesting that we found more class I related gene sequences than there are class I products on the genetic map. A similar distribution of class I genes in the BALB/C mouse ($H\text{-}2^d$) was described by Steinmetz et al. [9]. There are not enough class I genes in the *H-2* region to account for all different alleles.

To examine which of the cosmid clones contains a functional gene we introduced all the cosmids into mouse L cells [10] and selected for stable, thymidine-kinase-positive transformants. We tested for expression of new $H\text{-}2^b$ cell surface antigens on the transformed cells using the following assays: (a) direct monoclonal and alloantibody binding; (b) antibody-dependant complement-mediated lysis; (c) alloreactive anti-$H\text{-}2K^b$ and anti-$H\text{-}2D^b$ T-cell mediated lysis (CML); (d) $H\text{-}2K^b$-

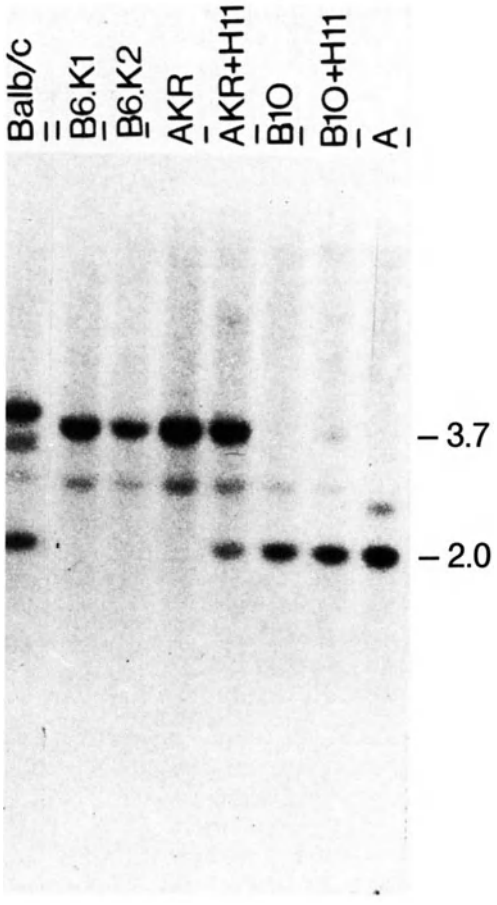

Fig. 3a. Chromosomal blot localizing region *6;* DNA was digested with PstI and probed with the 2.0-kb Eco-fragment from region *6.* In lanes indicated, 100 pg cosmid HII and 5 μg chromosomal DNA was digested with Pst I

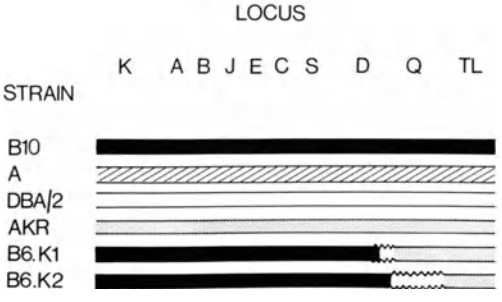

Fig. 3b. Genetic map of the recombinant strains. *Jagged section* indicates area where chromosome is derived from either B10 or A

Table 2. Immunoselection assays for L cells transformed with cosmids containing class I genes

Assay	Ltk$^-$	B10	LH8	LH8EcoB	LH25	LH11	LH1.1
Antibody binding							
Anti-H-2^k	+	–	+	+	+	+	+
Anti-H-2K^b	–	+	+	+	–	–	–
Anti-H-2D^b	–	+	–	–	–	–	+
Antibody-dependant cytotoxicity							
Anti-H-2^k	+		+		+		
Anti-H-2^b	–		+		–		
Anti-H-2K^b	–		+		–		
Anti-H-2D^b	–		–		–		
Allogenic cytotoxic T-cell killing							
Anti-H-2^k	+	–	+	+	+	+	
Anti H-2^b	–	+	+	+	–	–	
T_c-cell-mediated killing of transformed L cells infected with influenza virus							
H-2K^b-restricted	–	+	+				–
H-2D^b-restricted	–	+	–				+

Ltk, mouse L cells (H-2^k) used for transfection experiments; B10, spleen cells of B10 mouse, H-2^b control cells; LH8, L cells transformed with cosmid H8 containing the *A* and *B* gene of region *1*; LH8EcoB, L cells transformed with an Eco subclone of H8, containing only the *B* gene of region *1*; LH25, L cells transformed with cosmid H25 containing the *A* gene of region *1*; LH11, L cells transformed with cosmid H11, containing the *A, B,* and *C* genes of region *6*; LH1.1, L cells transformed with cosmid H1.1 coding for the *H-2D^b* gene of region *2*

and H-2D^b-restricted influenza-virus-specific T-cell killing.

To date we have obtained positive expression data only for the *H-2K^b* [10] and *H-2D^b* [11] genes. The results are shown in Table 2. The newly expressed H-2 antigens are not only recognized by allo- and monoclonal antibodies, they also retain their in vivo function. They are recognized by alloreactive cytotoxic T cells and act as restriction elements for T_c-cell-mediated killing of influenza-virus-infected cells. The

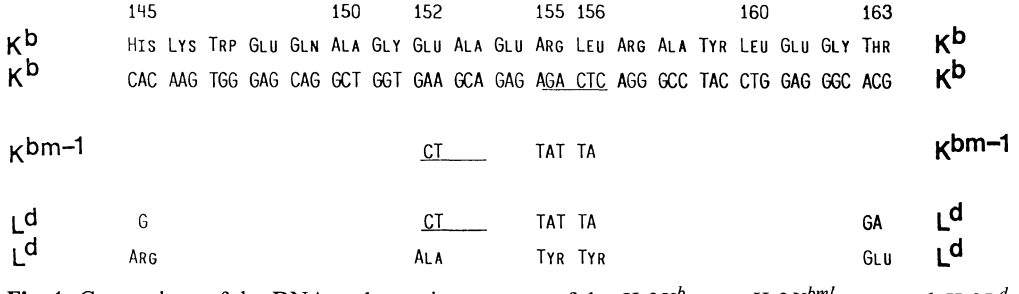

Fig. 4. Comparison of the DNA and protein sequence of the *H-2K^b* gene, *H-2K^{bml}* gene, and *H-2L^d* gene [19, 20] starting from amino acid 145 to 163. The recognition sequences for the enzyme PstI and Hinf I are *underlined.* Only the nucleotides and amino acid differences from *H-2K^b* are shown in the *H-2K^{bml}* and *H-2L^d* gene

gene *B* of region *1* codes for the *H-2K*^{b} antigen and the single gene of region *2* is the *H-2D*^{b} gene. The fact that the full complement of epitopes normally associated with the H-2K^b and the H-2D^b molecule is present on the surface of transformed cells makes it possible to analyse which determinants are recognized by antibodies and the T-cell receptor. Specifically, it should now be possible to transform L cells with hybrid *H-2* genes constructed in vitro.

We [12] and others [13] proposed gene conversion as a mechanism of generating different *H-2* alleles. Gene conversion is a mechanism by which genetic information can be transferred from one gene to another related gene; it was first described in fungi [14, 15]. One way to obtain information about the mechanism that generates polymorphism is to compare the DNA sequences of allelic genes. As the alleles are presumably the result of many gene conversion events, it might be difficult to trace individual sequences in the mosaic genes. We therefore decided to analyse a very recent allele, which was detected in one of the Bm- mutants [16]. These mutant mice (*H-2*^{b} haplotype background) express a changed H-2K antigen.

We cloned the *H-2K*^{bml} gene [17], the antigen it codes for was shown by peptide analysis to contain Tyr-Tyr at position *155* and *156* instead of Arg-Leu. We sequenced the *H-2K*^{b} gene [18] and the translated regions of the *H-2K*^{bml} gene. We found in total seven bp changes (Fig. 4) in a short region of 13 nucleotides, two result in another amino acid substitution as well as the two amino acid changes at positions *155* and *156*. Our data show that these changes occurred in the *H-2K*^{b} gene. The same sequences are found in the *H-2L*^{d} gene [19, 20]. We consider that a gene conversion event is the most likely cause for this phenomenon. In this case, there must be a donor gene that contains this novel nucleotide sequence. We are currently searching for a donor gene. We think it likely that gene conversion will turn out to be a major force if not *the* major force in the generation of new alleles of *H-2* genes.

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Presence of HTLV in a Subset of T Cells from an Infected Patient: Some Immunochemical Properties of the Infected Cells

M. S. Reitz, D. Mann, M. F. Clarke, V. S. Kalyanaraman, M. Robert-Guroff, M. Popovic, and R. C. Gallo

We have previously reported the isolation of a retrovirus (HTLV for human T-cell leukemia-lymphoma virus) from a patient with cutaneous T-cell lymphoma [3]. HTLV was shown to be unrelated to previously described retroviruses and not endogenous to humans, and integrated proviral DNA was present and expressed into mRNA in the malignant T cells of the same patient [5]. A closely related virus was subsequently isolated from a patient with cutaneous T-cell leukemia-lymphoma [4]. Since then we (Popovic et al., in preparation; Gallo et al. and Sarin et al., this volume) and others [2] have also isolated virus from a number of other patients. All appear quite closely related to the first HTLV isolate (Popovic et al., submitted; Gallo et al., this volume).

Subsequent to the discovery of HTLV in two separate T-cell lines from the first patient (CR), we became aware of the existence of independently derived T- and B-cell lines from the same patient, established in Dr. T. Waldmann's laboratory. This gave us the opportunity to determine the distribution of an HTLV-infected patient. The cell lines used in this study include: HUT-102, a TCGF-independent T-cell line established from the peripheral lymph node of CR using purified TCGF [4]; clones B2 and A9, TCGF-independent sublines of HUT-102 [1]; CTCL-3, TCGF-dependent T-cell lines established from the peripheral blood of CR using purified TCGF; CTC-16, TCGF-dependent T-cell line established from the peripheral blood of CR using crude TCGF [7]; C6 and G5, TCGF-dependent sublines derived from CTC-16 by the limiting dilution method; and CR-B, a B-cell line derived from peripheral blood after immortalization with Epstein-Barr virus strain B-95. The presence of HTLV was assayed in at least one of several ways: (1) p19 was assayed by indirect immunofluorescence using a murine monoclonal antibody; (2) p24 was assayed with a RIP assay using goat hyperimmune sera (see Sarngadharan et al., this volume); (3) RNA and (4) DNA were assayed by liquid hybridization as described by Reitz et al. [5].

HTLV was abundantly present in the HUT-102 line and the derived clones B2 and A9 (Table 1). The great majority (~90%) of these cells expressed p19, and provirus was present at three to four copies per haploid genome. CTCL-3 also contained HTLV, since 15%–20% of these cells

Table 1. Surface markers of HTLV-infected cells

Cell line	Virus	OKT markers (% cell+)				
		3	4	6	8	10
T cells						
Clone B2	++	2	90	5	2	2
Clone A9	++	5	70	5	5	ND
CTCL-3	+	7	94	15	15	19
CTC-16	+	29	86	12	6	5
Clone C6	±[a]	75	5	10	2	4
Clone G5	±[a]	26	5	ND	62	ND
B cells						
CR-B	–	5	5	75	5	5

ND, not done
[a] No p19 or proviral DNA detectable. Low levels of p24 present

Table 2. HLA typing of cell lines derived from patient CR

Cell lines	HLA-A	HLA-B	HLA-DR and MB
T cells			
HUT-102	Aw30, Aw31 (A10), (A11), (A29)	B17, B18 (Bw35), (Bw50)	DR2, DRw6, MB
B2 (HUT-102)	Aw30, Aw31 (A10), (A11), (A29)	B17, B18 (Bw35), (Bw50), (B12)	DR2, DRw6, MB
A9 (HUT-102)	Aw30, Aw31 (A10), (A11), (A29)	B17, B18 (Bw35), (Bw50), (B7)	DR2, DRw6, MB
CTCL-3	Aw30, Aw31 (A10), (A11)	B17, B18 (Bw35)	DR2, DRw6, MB
G5 (CTC-16)	Aw30, Aw31 (A10), (A11), (A29)	B17, B18	DR2, DRw6, MB
C6 (CTC-16)	Aw30, Aw31	B17, B18	DR2, DRw6, MB
B cells			
CR-B	Aw30, Aw31	B17, B18	DR2, DRw6, MB

express p19. In contrast, the B-cell line (like normal human T cell) does not express either viral protein. Viral RNA and proviral DNA are not detectable even under conditions which would detect one proviral copy per eight haploid genomes.

CTC-16 also contains HTLV-infected cells since 35%–40% of the cells express p19. C6 and G5, derived from CTC-16, are negative for both p19 and proviral DNA (to a Cot of 20,000). Low levels of p24 are detectable (~1% of that observed with B2 and A9). Our interpretation of this data is that C6 and G5 are not clonal with respect to HTLV provirus and that only a few cells are infected and producing protein. Since the p24 assay is more sensitive than the p19 assay under the conditions used, low levels of p19 expression could give a negative result.

All the cell lines were analyzed for surface markers with a FACS II cell sorter. The cells within the cultures with high levels of HTLV tended to be OKT $4^+3^-6^-8^-10^-$ (Table 1). C6, which contained mostly uninfected cells, was predominantly OKT $3^+4^-6^-8^-10^-$, and G5 (also mostly containing uninfected cells) was largely OKT $8^+3^-4^-$. Thus, in patient CT, HTLV appears to be mostly present in the peripheral blood in a particular set of T cells.

All the cell lines used were typed for HLA expression in order to verify that they originated from CR. All had the same HLA-DR haplotype as the patient and all expressed HLA-Aw30 and Aw31 and HLA-B17 and -B18, consistent with the haplotype of fresh cells from CR and with that of his parents (Table 2). However, all the infected T-cell lines expressed extra, inappropriate HLA determinants on all of the cells, as judged by microcytotoxicity assays. This has now been observed with infected T cells from six different patients and with cord blood T cells infected with HTLV in vitro. DNA was purified from T and B cells, and digested with different restriction enzymes. We then hybridized these DNA digests to labeled HLA cloned DNA [6], by the Southern blot technique. Most restriction patterns for the two DNAs were similar, indicating that there are no massive rearrangements of the HLA gene complex. However, the patterns differed after digestion with *HpaII* and *XhoI*. Since these enzymes are inhibited by DNA methylation, this suggests that the band T cells differ with respect to the degree of methylation of their HLA class I genes. Whether this is due to virus infection is a question of obvious interest. The exact nature of these possible methylation differences, as well as of the proteins which

carry the apparently inappropriate HLA determinants, is currently under investigation.

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Hybrids Between Human Cell Lines Belonging to Different Hematopoietic Pathways: Analysis of HLA and Myeloid Surface Antigens

A. Ziegler, B. Uchańska-Ziegler, P. Wernet, and J. Zeuthen

A. Introduction

The control of gene regulation can be examined by somatic cell hybridization. Hybrids between human cell lines belonging to different hematopoietic lineages provide model systems for the analysis of the mechanisms governing the expression of cell surface antigens specific for a particular differentiation pathway [3, 4, 12]. In this study, the expression of antigens encoded by the human major histocompatibility (HLA) complex and of molecules present on myeloid cell types were analyzed with monoclonal antibodies on two somatic cell hybrids, HP-1 and PUTKO1.

B. Materials and Methods

HP-1 [4] was produced by fusing the Burkitt's lymphoma-derived B-cell line P_3HR-1 [3] and HL-60 [2], which is a promyelocytic leukemia-derived cell line. PUTKO was a somatic hybrid obtained by fusing P_3HR-1 and K562 [5], a fetal erythroid cell line [8]. All five cell lines were grown in tissue culture medium containing 10% fetal calf serum and antibiotics. Most of the monoclonal antibodies employed in this work have been described before (see Table 1). The expression of cell surface antigens recognized by monoclonal antibodies was determined using bacterial binding assays [11, 15].

Table 1. Monoclonal antibodies and their specificities

Monoclonal antibodies	Cell type or antigen detected	References
W6/32.HL	HLA-A, B, C heavy chains	[1]
W6/32.HK	Inactive variant	[13]
TÜ48	HLA-Aw23, -Aw24, -Aw32, -Bw4	[6]
2BC4	HLA-Bw6	Westphal, unpublished
TÜ22, TÜ34, TÜ35, TÜ36, TÜ37, TÜ39, TÜ43, TÜ58,	Ia-like antigens	[15]
YD1/63. HLK		[7]
TÜ3, TÜ50, TÜ51	Myeloid cells	[11]
TÜ5, TÜ6, TÜ9	Myeloid cells	[9]
TÜ8	Myeloid, some monocytoid and certain T and B cells	[11]
TÜ12	T-cell subset, immature myeloid cells	[11]

C. Results and Discussion

I. Antigens Encoded by the HLA Complex

All cell lines examined here expressed HLA heavy chains as detected by W6/32.HL (Table 2), a finding in line with previous results [3, 4, 12, 14]. The supertypic antigenic determinant HLA-Bw4, defined by TÜ48, was present on HL-60, P_3HR-1, and their hybrid HP-1 but lacking from K562 and the K562 × P_3HR-1 hydrid PUTKO1. An analysis of HLA antigen expression on DUTKO1, another K562 × B cell hybrid [12, 15] also indicated that K562 and hybrids derived from it have a deficiency in the expression of HLA-B antigens. These results make it likely that HLA-A,C, and HLA-B molecules are under separate genetic control. This situation seems to apply also to thymic cells, since HLA-B molecules are not detectable on cortical thymocytes, although these can be shown to express to other type(s) of HLA heavy chains (Müller et al., unpublished).

Table 2. Expression of major histocompatibility complex-controlled antigens by the hybrids and their parental cells

Antigen detected by	Cell line				
	HL-60	HP-1	P_3HR-1	PUTKO1	K562
W6/32.HL	100%[a], ~80[b]	100%, ~55	100%, ~60	80%, ~20	95%, ~15
W6/32.HK	–	–	–	–	–
TÜ48	100%, ~80	100%, ~30	100%, ~50	–	–
2BC4	NT[c]	NT	NT	–	–
TÜ22	2%, ~5	65%, ~20	78%, ~20	–	–
TÜ34	–	100%, ~40	98%, ~35	–	–
TÜ35	6%, ~5	94%, ~25	91%, ~35	≪1%, ~20	–
TÜ36	–	98%, ~40	94%, ~35	1%, ~50	–
TÜ37	–	99%, ~40	85%, ~25	<1%, ~30	–
TÜ39	7%, ~5	95%, ~25	92%, ~35	1%, ~30	–
TÜ43	–	100%, ~40	91%, ~35	<1%, ~20	–
TÜ58	2%, ~5	100%, ~40	86%, ~25	<1%, ~25	–
YD1/63.HLK	–	100%, ~30	73%, ~40	NT	–

[a] Percentage of cells with three or more bacteria bound
[b] Average number of bacteria bound per cell
[c] NT not tested

Table 3. Expression of "myeloid" antigens by the hybrids and their parental cells

Antigen detected by	Cell line				
	HL-60	HP-1	PUT	PUTK01	K562
TÜ3	35%[a], ~ 10[b]	–	–	–	–
TÜ5	90%, ~ 60	–	–	–	–
TÜ6	90%, ~ 70	–	–	–	–
TÜ8	98%, ~ 90	21%, ~30	–	–	8%, ~ 9
TÜ9	98%, ~100	26%, ~30	–	–	19%, ~35
TÜ12	68%, ~ 15	40%, ~10	–	–	–
TÜ50	92%, ~ 70	9%, ~10	–	–	–
TÜ51	92%, ~ 90	–	–	–	–

[a] Percentage of cells with three or more bacteria bound
[b] Average number of bacteria bound per cell

In P_3HR-1 hybrids, whether the cells express Ia-like antigens seems to depend on the fusion partner. These molecules were present on virtually all HP-1 cells (with the exception of TÜ22 molecules), and could be detected even on a very small subpopulation of PUTKO1 cells. The postulated "dominance" of the K562 genome in a K562×B cell hybrid [3] is thus not complete, since Ia-like antigens continue to be expressed on some hybrid cells, which have therefore retained at least one characteristic surface marker from their parental B cell.

II. "Myeloid" Antigens

These antigens were expressed by HL-60 cells, but not by the B-cell line P_3HR-1, while K562 cells only showed reactivity with the antibodies TÜ8 and TÜ9. Although HP-1 hybrid cells seem to have lost all functional attributes of their myeloid parent HL-60 [4], they appeared to retain certain "myeloid" surface antigens, as shown in Table 3. A preliminary study of several clones from HP-1 cells (Zeuthen and Ziegler, unpublished) shows that it may be possible to obtain clones which do not bear most of the antigens characteristic for the myeloid cell types detected here. On the other hand, PUTKO1 cells appeared to have lost the ability to express the antigens detected by TÜ8 and TÜ9, although they are much more similar to K562 than to their other parent [3].

Since these antigens are glycosphingolipids (Towbin and Ziegler, unpublished), it may be of interest to examine the activity of glycosylases and glycosyltransferases in the hybrid cell lines employed here.

The results make it likely that gene dosage effects cannot be solely responsible for the observed phenomena. Furthermore, the phenotype of a hybrid cell cannot be predicted with certainty from the properties of the parental cells.

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Natural Killer Cells and Their Targets: Impact of Differentiation on Target Cell Susceptibility*

M. Gidlund, K. Nilsson, T. Tötterman, and H. Wigzell

A. Introduction

Natural killer (NK) cells display selective binding/lytic properties insofar that they kill only certain target cell types. Although the recognition structures of NK cells and their respective targets are still largely unknown, accumulated data indicate that target susceptibility may be determined in part by the stage of differentiation of the target cells [1–3]. Thus, natural killer cells can frequently easily kill cells, both malignant as well as normal cells, at earlier "primitive" stages of differentiation. We have used this approach in testing cloned tumor cell lines undergoing controlled differentiation as an assay system for exploring the differentiation-related events in more detail. The present article summarizes our present state of knowledge as achieved by these studies.

B. Material and Methods

Basically our approach was to use a ^{51}Cr-release assay using various target cells, notably in vitro growing tumor cell lines as described previously [4]. Differentiation was induced under varying conditions and with widely different agents, and the consequences of differentiation were analyzed by a variety of markers including NK susceptibility. Changes in resistance to NK effector cells in the respective targets were in several cases also tested in relation to changes in the ability of the targets to function in cold target inhibition assays [5]. In all the differentiation systems studied the consequences of induction of differentiation on kinetics were followed, and any direct impact of the inducing agent on NK susceptibility of the targets was excluded.

C. Results

I. Tumor Systems Where Differentiation Leads to Resistance to NK Lysis

In our initial studies a series of tumor systems studied were all found to express an increase in NK resistance in parallel to the induced differentiation [1]. This was regardless of agent(s) used to induce differentiation. In most systems the resistance to lysis was accompanied by a parallel reduction in the capacity of the differentiated cells to function as cold target inhibitors in NK lysis. Our data obtained were thus compatible with the notion that NK cells tended to react against cells in the earlier stages of differentiation. Cells at a later stage of differentiation would be relatively deficient in their ability to react with NK cells, this probably accounting for a major (total?) part of the increase in NK resistance. The tumor systems studied included widely different types of cells such as teratocarcinomas, histiocytomas, and myeloid leukemias. A summary of the results obtained is shown in Table 1. In some of the tumor systems it was possible to obtain clones of cells of "spontaneous" origin with markers indicating increased differentiation. Such clones obtained without any

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Table 1. Tumor systems where induced differentiation leads to a decrease in NK susceptibility

Cell type	Cell line	Inducer	Differentiation markers	NK sensitivity
Erythroid leukemia (Human)	K562	Sodium butyrate or hemin	Glycophorin, hemoglobin	Decreased
Histiocytoma (Human)	U937	TPA	Fc receptors, HLA-DR	Decreased
Erythroid leukemia (Mouse)	GM-86	HBMA	Hemoglobin, surface proteins	Decreased
Embryonic carcinoma (Mouse)	PC13	Retinoic acid	Endodermal differentiation	Decreased

known added inducing agent displayed the expected decrease in NK susceptibility, as shown in Table 2. This showed therefore that the decrease in NK sensitivity indeed seemed to be linked to differentiation stages regardless of how this stage was reached/induced in the particular tumor under scrutiny.

II. Tumor Systems Where Differentiation Leads to Susceptibility To NK Lysis

Subsequent to the above studies we started to encounter tumor systems where induction to differentiation led to the opposite effects, namely an increase in NK susceptibility [3]. This was encountered in two systems, namely neuroblastoma cells in vitro and also when inducing differentiation in CLL cells freshly obtained from patients. Table 3 summarizes the results obtained in these two systems with regard to surface and morphological markers in relation to changes in NK susceptibility. It had previously been found that Burkitt lymphoma cells in general are more susceptible to NK lysis than the corresponding EBV-transformed B-cell lines [6]. The present studies thus add CLL cells to the category of cell types within the B-cell series that could be attacked by NK cells, in particular if induced to differentiate. In-

Table 2. Examples of "spontaneously" differentiating K562 cells expressing expected decrease in NK sensitivity

Cell type	Marker used for cloning	NK sensitivity
K562	–	50.0
K562, clone 4	Increased glycophorin	37.9 ($P > 0.025$)
K562, clone 6	Increased glycophorin	29.5 ($P > 0.001$)

Clones produced via agar cloning and screening for surface glycophorin-positive clones. Figures denote % specific release of ^{51}Cr mediated by NK cells at 100 : 1 ratio

Table 3. Tumor systems where induced differentiation leads to an increase in NK sensitivity

Cell type	Cell line	Inducer	Differentiation markers	NK sensitivity
Neuroblastoma	SH-SY5Y	TPA	Catecholeamines Nerve-specific enolase	Increased
CLL	Fresh cells	TPA	Cytoplasmic Ig	Increased

Table 4. Positive correlation between ability of TPA to induce NK susceptibility in CLL cells and active stage of disease

		Progressive disease	
		Yes	No
Increase in NK sensitivity[a]	Yes	14	2
	No	1	14

[a] Yes statistically increased NK susceptibility after TPA treatment. Figures denote number of CLL patients studied

terestingly, the ability of CLL cells to undergo differentiation leading to an increase in cytoplasmatic Ig and NK susceptibility could be shown to be positively correlated with the disease being in an active stage [3]. This correlation was found highly significant, as indicated in Table 4, where the aility to become NK susceptible could even be used as an indicator of imminent onset of active disease in patients with stable CLL disease.

D. Discussion

In the present article we have briefly summarized our evidence that NK cells may be able to bind and lyse certain targets depending at what stage of differentiation that particular cell is located at the time of the assay. The picture has some general features but also some quite sizeable controversial and complicated aspects. Thus, it is clear that NK cells may indeed have a general tendency to be aggressive against more undifferentiated cells within a cell lineage [1–3]. However, within a particular cell lineage sizeable variation may exist between cells at different stages of differentiation. Whereas bone marrow stem cells may belong to the NK susceptible "pool" [7] lymphoid cells of B-cell nature may or may not be susceptible. Thus, EBV lymphoblastoid cells are in general fairly resistant to NK lysis like most freshly obtained CLL cells [3]. Also, both NK-resistant as well as NK-susceptible cells may be found within human myeloma cells/cell lines, that is using cells supposedly very highly differentiated within this lineage [6]. One would then have to conclude that the NK susceptible cells within the B-cell lineage (mostly Burkitt-lymphoma cell lines, TPA-induced CLL cells from patients in active disease, and myeloma cells from certain patients) do not follow a safe and steady change in one direction only with regard to NK sensitivity upon differentiation. This could either be explained on the assumption that certain differentiation markers on B cells that can serve as NK target moieties may be able to express themselves at more than one time during differentiation of B cells. Alternatively, there may be some surface changes linked to differentiation which in more general terms may allow a cell type to change in a + or – fashion with regard to NK sensitivity. In particular this would seem to be the case for certain glycolipid changes where so far in the presently studied differentiation systems there has always been a positive correlation found between the change in concentration of these glycolipids (and their degree of sialic acid conjugation) and the corresponding changes in NK susceptibility [8]. The same differentiation-inducing agent, e.g., TPA, could be shown here to have an opposite impact on glycolipid composition in the two respective tumor groups (=displaying increase or decrease in NK sensitivity upon differentiation). Further analysis would be required to analyze whether these glycolipid changes cause the observed changes in susceptibility to NK lysis or whether they are merely a side phenomenon of no direct relevance.

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Natural Cellular Defense Activities Against Tumors – Cytostasis and NK Activity*

R. Ehrlich, M. Efrati, B. Gonen, L. Shochat, and I. P. Witz

Several immunocyte populations are active in the natural cellular defense against tumors. Among these are macrophages [1], natural killer (NK) cells [2], cells mediating antibody-dependent cellular cytotoxicity [3], natural cytotoxic (NC) [4] cells, and a variety of tumor cells. The NK cells kill mainly lymphoid tumor cells, and the cytostatic activity is directed against adherent tumor cells which originate from solid tumors.

In the present study we describe murine cytostatic activity and several physical and biological characteristics of the spleen cell populations mediating it.

Table 1. Comparison of cytostasis and NK activity

	Cytostasis	NK activity
Adherence (Sephadex G10)	+ and –	–
Phagocytosis	–	–
Thy-1	–	–
Fcγ-receptor	+	–
Activity in:		
10-day-old mice	Fully expressed	Lower than young adults
12-month-old mice	Fully expressed	Lower than young adults
Effect of:		
Incubation at 37 °C	No effect	Activity disappears
Hydrocortisone acetate (in vivo)	No effect (or enhancement)	Decreased activity
Carrageenan (in vivo)	No effect (or enhancement)	Decreased activity
LPS (in vivo)	Increased for longer than 5 days	Increased for 48 h
dsRNA	No effect	Increased activity
Effect of primary tumor bearing:		
Urethan induced	Decreased	No change
DMBA induced	Increased	Decreased
Induced by forced breeding	No change	Decreased

natural cytostatic cells [5]. The activities of these cells are directed against membrane determinants which are widely spready on

Table 1 summarizes some characteristics of natural cytostatic cells in comparison to NK cells. It can be seen that the only common feature of these two populations is the absence of a thymic (thy-1) antigen and that both types of cells are nonphagocytic.

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Table 2. Fractionation of splenocytes from normal mice according to adherence

	NK activity		
	Unfractionated	Nonadherent	Adherent
% cells in fraction	100	37	7
No. of lytic units/10^7 effectors	30	15	7.5
No. of lytic units/fraction	30	5.5	0.5
	Cytostatic activity		
% cells in fraction	100	37	7
No. of cytostatic units/10^7 effectors	5	5	10
No. of cytostatic units/fraction	5	5	0.7

Table 3. Fractionation of splenocytes from mice bearing primary DMBA-induced tumors according to adherence

	NK activity		
	Unfractionated	Nonadherent	Adherent
% cells in fraction	100	43	9
No. of lytic units/10^7 effectors	0	0	0
No. of lytic units/fraction	0	0	0
	Cytostatic activity		
% cells in fraction	100	43	19
No. of cytostatic units/10^7 effectors	10	2.5	10
No. of cytostatic units/fraction	10	1.1	1.9

Utilizing different adherence properties of NK and cytostatic cells on Sephadex G10 columns murine splenocytes can be separated into a nonadherent population which expresses most of the NK activity and an adherent population which when eluted expresses most of the cytostatic activity (Table 2). Cytostatic cells and NK cells respond differently to the bearing of primary adenocarcinomas and adenoacanthomas induced by the chemical carcinogen dimethybenzanthracene (DMBA). The NK activity in tumor-bearing mice decreases to zero levels while the cytostatic activity increases considerably (Table 3). The data clearly show that there is an enrichment in the cytostatic activity in the adherent cell fraction of tumor-bearing mice.

The fact that the cytostatic activity is boosted in mice-bearing tumors is of much potential interest, and these findings should be extended to other tumor systems including cancer in humans.

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Structure and Function of the Transferrin Receptor – A Possible Role in the Recognition of Natural Killer Cells

L. Vodinelich, C. Schneider, D. R. Sutherland, R. A. Newman, and M. F. Greaves

The monoclonal antibody OKT9 reacts specifically with the receptors for transferrin in human cells and has been used to isolate and characterise this receptor [1]. The receptor is a dimeric glycoprotein (Mr = 180,000) composed of two apparently identical subunits (Mr = 90,000) which are disulphide linked. The transferrin receptor appears to be a transmembrane molecule and is phosphorylated, the phosphate group being predominantly on serine residues. The cell surface form of the molecule possesses both complex and high mannose oligosaccharide chains, which do not appear to have a direct role in antibody (OKT9) binding. The molecule can be cleaved from the cell surface into a 70,000 molecular weight fragment, suggesting that the major part of the receptor is exposed to the extracellular environment. The released 70,000 molecular weight fragments are not disulphide linked and possess antibody (OKT9) binding sites. Cross-linking studies using radiolabelled transferrin suggest that two molecules of transferrin are bound to each 180,000 molecular weight receptor dimer. In addition, each 70,000 molecular weight fragment can independently bind one molecule of transferrin (Fig. 1).

We have observed that normal or malignant cells generally regarded as sensitive targets for so-called NK cells [2], e.g. cer-

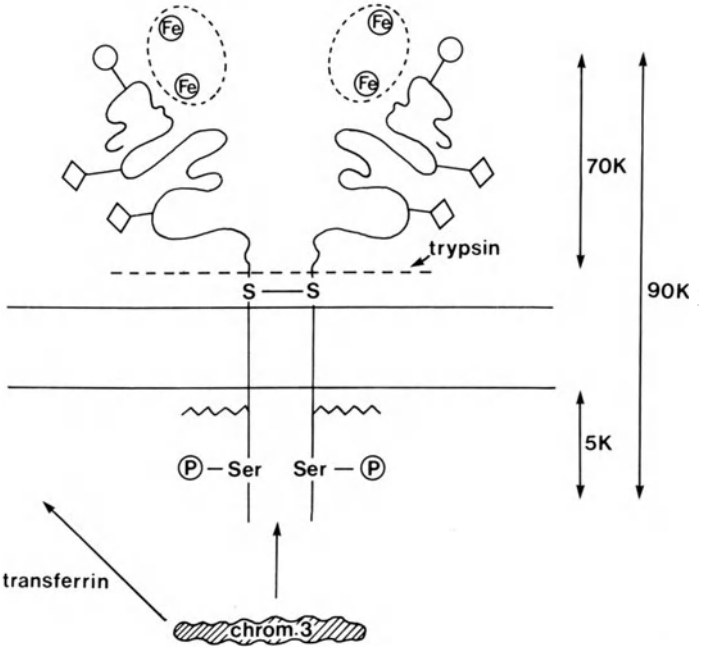

Fig. 1. Independent binding of the 70,000 molecular weight fragment to one molecule of transferrin. See [4] and [5] for details

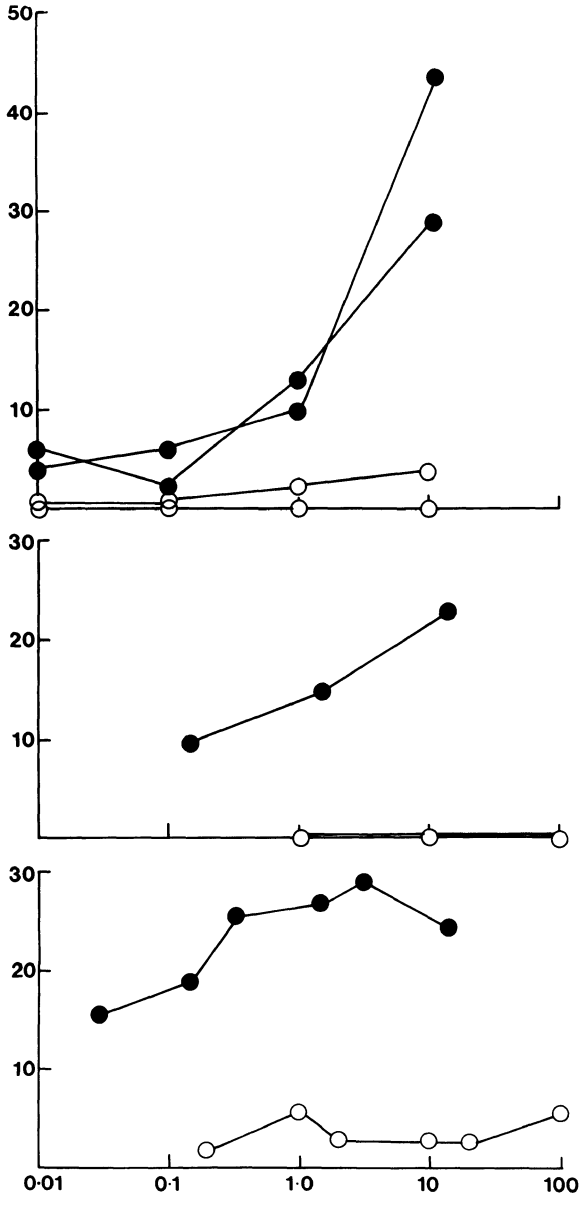

Fig. 2. Inhibition of NK activity by the purified trf receptor or purified HLA-AB molecules. *Vertical axis,* percentage of inhibition of ^{51}Cr release from K562 target cells; *horizontal axis,* concentration of trf receptor or HLA-AB molecules (μg/ml) in the assay mixture. ○—○, HLA-AB; ●—●, trf receptor. Experiments 1A and 1B were done using the first batch of trf receptor and HLA-AB antigen on two separate occasions. E : T ratio = 100 : 1. Experiments 2 and 3 were done using the second batch of trf receptor and HLA-AB antigen. Experiment 2, E : T = 100 : 1; Experiment 3, E : T = 25 : 1

tain leukaemic cell lines such as K562 and T leukaemic lines, fetal thymocytes, haemopoietic progenitor cells, had a strong expression of transferrin receptors, whereas cell types that are found to be insensitive to NK attack, e.g. resting lymphocytes, some B-cell lines, have low or negligible transferrin receptor levels. This prompted us to investigate the possibility that trf receptors represent recognition structures for NK cells. Three approaches were adopted: (a) analysis of the correlation between sensitivity to natural killing and the proportion of trf receptor positive cells in different cell lines; (b) a study of the relationship between levels of trf receptor expression in cell lines and their capacity to inhibit, competitively, recognition and killing of the target cell K562 by NK cells and (c) a comparison of affinity purified, soluble, proteolytic fragments of trf receptor and HLA-AB molecules for their ability to inhibit the natural killing effect.

In initial experiments we tested human malignant and non-malignant cell lines for their sensitivity to killing by fresh human

mononuclear cells from peripheral blood, using the 4-h cytotoxicity assay according to Herberman and Holden [2]. The same target cells were tested for their expression of trf receptors using the monoclonal antibody OKT9, which was reacted with goat anti-mouse FITC ($F(ab')_2$). A pattern of NK sensitivity emerged, which divided those cell lines which had a high proportion of trf receptor positive cells into three categories: NK sensitive (40%–55% lysis), NK weakly sensitive (10%–40% lysis) and NK insensitive (1%–10% lysis). Those cell lines which weakly or negligibly expressed trf receptors were minimally sensitive or, mainly, resistant to NK cells.

Specificity of the recognition by NK cells was investigated by 'cold' target inhibition assay [3]. The capacity of test cell lines to compete with the ^{51}Cr-labelled K562 cell as targets for NK effectors and thus cause inhibition of ^{51}Cr release from K562 was compared with the competitive capacity of unlabelled K562 cells. The same batches of cells were tested for the presence of trf receptor using OKT9 and I^{125} goat anti-mouse Ig as a second layer antibody. In attempting to correlate inhibitory capacity of cells with trf receptor expression, we took the inhibitory capacity of 10^6 test cells expressed as a proportion of the activity detected with the same number of K562 inhibitory cells. Linear regression analysis indicated a direct correlation between the two parameters ($r=0.86$, $P<0.0005$) for cell lines having fewer trf receptors than K562. Significantly, however, cell lines with a higher total content of trf receptors than K562 did not express a greater inhibition capacity, giving an overall linear correlation with a corresponding $r=0.698$ ($P<0.0005$). This suggests that K562 could have some other advantage (for example, size, morphology, charge, hydrophobicity) as a competitor with itself in the assay.

Definitive involvement of trf receptors in NK recognition was tested by blocking the NK-mediated lysis of K562 cells with a 70K fragment (trypsin cleaved) of the receptor. A 38K HLA-AB fragment (papain cleaved) was used as control. The results from four similar experiments are shown in Fig. 2. The trf fragment partially inhibited the NK-induced lysis of K562 cells, whilst no significant inhibition was observed in samples incubated with the HLA-AB antigen even when the concentration of the HLA-AB molecules was ten times higher than the concentration of the trf receptor.

These experiments provide data compatible with our hypothesis. Thus, the existence of target recognition structures and the trf receptors are correlated. In addition, purified trf receptors could inhibit NK cytotoxicity. Incomplete inhibition may be due to the large degree of denaturation of the fragments, their rapid internalisation by the cells in the assay, lower avidity and/or the presence of additional target recognition structures on K562 cells.

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NK and K Cells in Malignant Lymph Nodes

J. Milleck, P. Jantscheff, F. Irro, N. Tkatscheva, and Lê Dinh Hóe

A. Introduction

Natural killer (NK) cells and effector cells of the antibody-dependent cellular cytotoxicity (K cells) show a striking organ distribution. Above all they occur in blood, bone marrow, and spleen. Cells of other lymphoreticular organs display a very low, if any, spontaneous killer cell activity (Table 1). Normal lymph node cells are nearly inactive [1, 2, 8, 14]. There are no data, however, on to what extent lymph node cells from lymphoma patients have killer cell activity. It is conceivable that the expansion of malignant transformed lymph node cells gives rise to functionally different cells. Tests with blood or bone marrow cells from patients suffering from acute lymphoblastic or acute nonlymphoblastic leukemia suggest the existence of malignant blasts with NK- or K-cell activity [3, 6, 7].

B. Patients and Methods

Basic data of four nontreated patients suffering from a non-Hodgkin lymphoma (NHL) or a lymphoma-like disease are shown in Table 2. Mononuclear leukocytes from axillary or inguinal lymph nodes (LK), peripheral blood (PBL), or bone marrow (KM) were prepared by Ficoll-visotrast centrifugation, and killer cell activities were estimated by the ^{51}chromium-release technique. Ten-thousand target cells in 100 µl were incubated with an excess of 50 times and 10 times the effector cells at 37 °C for 4 h. Targets were cells of the K-562 cell line for NK cell and mouse leukemia cells coated with rabbit antibodies for K-cell estimation.

Table 1. Distribution of NK and K cells in different lymphatic organs

Organ	n[a]	NK	n	K
Blood	118	38 (0 – 78)[b]	165	43 (0.5 – 81)
Bone marrow	11	35 (8 – 69)	14	43 (14 – 61)
Spleen	7	24 (9 – 92)	9	33 (12 – 72)
Lymph node	18	4 (0 – 13)	15	2 (0 – 4)
Tonsil	18	4 (0.5 – 11)	18	4 (1 – 8)
Thymus	10	1 (0 – 4)	10	1 (0 – 2)

Origin of organs: Blood (healthy donors), bone marrow (healthy donors or patients with cholecystitis), spleen (Hodgkin patients), lymph nodes (patients with intestinal solid tumors), tonsils (persons with tonsillitis), thymus (young children). Data refer to mononuclear leukocytes prepared by Ficoll-Visotrast centrifugation

[a] Number of donors tested

[b] Mean percentage of specific ^{51}Cr-release (range) at a ratio of effector: target cells of 50 : 1

Table 2. Basic data of the lymphoma patients

Patients	Age	Sex	Diagnosis	Clinical stage	Blood leukocytes/ mm^3
H.G.	64	m	Sezary syndrome	(III A)	4,550
K.B.	45	m	Angioimmunoblastic lymphadenopathy	III B	15,400
J.H.	52	f	Centrocytic-centroblastic lymphoma	III A	4,750
I.G.	46	f	Centrocytic-centroblastic lymphoma	IV A	5,250

C. Results and Discussion

Figures 1–5 illustrate the spontaneous killer cell activity of lymph node cells isolated from patients suffering from NHL. Figure 1 shows that the lymph node cells of a patient with Sezary syndrome were only K-cell active, but not NK-cell active. This finding is surprising insofar as there has been no example for K-cell active lymph node cells. The attempt to stimulate normal lymph node cells immunologically, e.g., by a lymphocyte-mixed culture, leads only to an increase in NK-cell similar activity, but not in K-cell activity [7].

In view of the anomalous behavior of the lymph node cells it may be assumed that the lymphoma cells themselves are functioning as K cells.

The Sezary syndrome is the leukemic variant of a cutaneous T-cell lymphoma [15]. The case described here resembles or

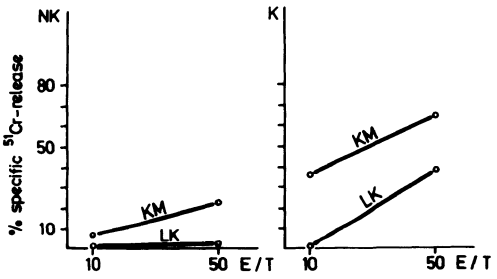

Fig. 1. NK- or K-cell activity of lymph node and bone marrow cells from a patient suffering from a Sezary syndrome

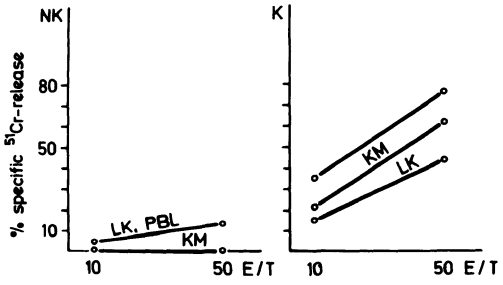

Fig. 3. The same patient as in Fig. 2; lymph nodes started rapidly regressing 2 weeks after discontinuing polychemotherapy

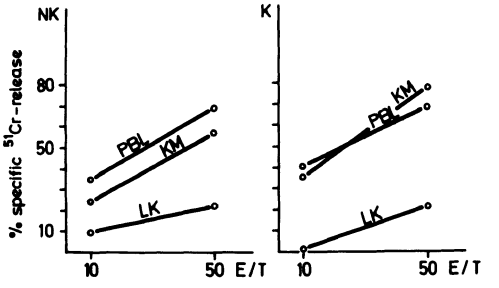

Fig. 2. NK- or K-cell activity of lymph node, peripheral blood, and bone marrow cells from a patient with an angioimmunoblastic lymphadenopathy

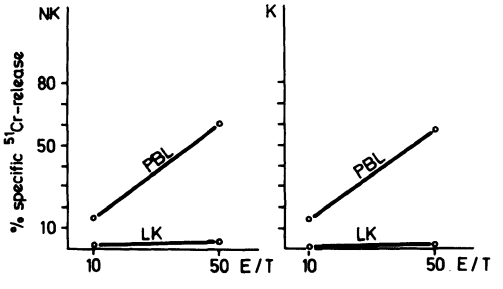

Fig. 4. NK- or K-cell activity of lymph node and peripheral blood cells from a patient suffering from a centrocytic-centroblastic lymphoma (Brill-Symmers)

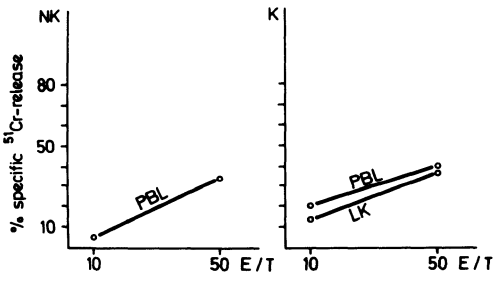

Fig. 5. NK- or K-cell activity of lymph node and peripheral blood cells from another patient with a centrocytic-centroblastic lymphoma

corresponds to those cases of a chronic lymphatic leukemia of the T-cell type in which T-lymphocytes from the blood have an immunosuppressive action, have Fc receptors for IgG and are either only K-cell active [9, 12] or both NK- and K-cell active [13]. Figure 2 shows the activity of lymph node cells of a patient with an angioimmunoblastic lymphadenopathy (AIL). The AIL or "lymphogranulomatosis X" is a lymphoma-like systemic disease, and may have rather different courses. Both spontaneous remissions and progression into a malignant lymphoma are possible [4, 5, 10, 11]. Because of the low NK- and K-cell activity of the lymph node, cells it was not possible to decide whether the spontaneous killer cells had arisen in the lymph node or had entered the lymph node through the blood stream. The patient had been treated with polychemotherapy. The lymph nodes swelled during treatment, but started to return rapidly to normal 2 weeks after discontinuing therapy. At this time, another lymph node was removed and studied. Figure 3 shows that the lymph node cells were K-cell active, but hardly NK-cell active. It is unclear whether there exists a relationship between the remission of the lymph nodes and the killer cell activity. The strongly reduced NK-cell activity of the blood and bone marrow cells seems to reflect the influence of chemotherapy. The activity of NK-cells is more strongly inhibited by chemotherapy than that of K cells [7].

The determination of functional and antigenic properties aims at finding a still more subtle classification of lymphomas and leukemias in order to obtain more prognostically relevant data. Estimation of the spontaneous killer cell activities may be a step in this direction.

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Contrasuppression, Class I Antigens, and Cancer Immunity*

D. R. Green and R. K. Gershon

When immunologic homeostasis is perturbed by antigen, the failure to accomodate that antigen as "self" usually results in what we observe as an immune response. In the case of tumors, however, a massive bombardment of the system with tumor antigens can induce a potent general suppression of immunity such that the tumor must often be removed before immunity can be demonstrated [1]. Such observations suggest that while there may be antigenic determinants on tumor cells which can serve as targets of immunity, immunoregulatory modification might be necessary for such immunity to become manifest. Thus, immune suppression induced by tumor challenge is probably the major stumbling block to effective immunity against many tumors. In terms of therapy, the activity of the suppressor circuit might serve as a target of effective immunengineering.

Alternatively, we can envision situations in which tumors of the lymphoid system may come under suppressor cell control and thus be rendered benign. For example, Rohrer and Lynch [2] have demonstrated control by suppressor T cells of MOPC-315 myeloma clone growth and secretion. Similar effects have been obtained by Abbas et al. [3]. Suppressor T cells appear in normal people infected with Epstein-Barr virus (EBV) [4], and such T cells have been shown to be capable of inhibiting in vitro transformation of B cells by EBV [5]. In some cases, therefore, *failure* to effect suppression of a proliferating cell may be a cause of cancer, so that therapy must then be aimed at enhancing suppressor cell activity. Such tumors, while rare, may be important for our development of effective tumor therapy.

The ability to up or down regulate immune responses is likely to be a key factor in cancer therapy. While the role of suppressor cells in cancer is an active area of research, little is known about the role of the cells that mediate contrasuppression. Contrasuppression is an immunoregulatory T cell activity which is defined functionally as the ability to interfere with suppressor cell signals. Contrasuppressor effector cells have been shown to express a unique profile of cell surface antigens and to function, at least in part, by rendering helper T cells resistant to suppressor cell signals. Clearly, such an activity could have major consequences for our understanding and control of cancer.

In this brief paper we will discuss the evidence that contrasuppressor T cells have an active role in the immune response to cancer. This will lead us into a hypothetical consideration of the role of class I antigens in the activation of regulatory T cells and the consequences of this theory for immunomodulation and therapy. Finally we will review evidence for the possibility that in some cases, involving transformed cells of the immune system, this regulatory activity might enhance tumor incidence by interfering with the suppressor cells capable of controlling tumor growth.

A. Immune Sequelae to the Activation of Contrasuppression

Relatively little is known about the nature of the signals which initiate contrasup-

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pression, but the dose of immunizing antigen is certainly a key factor. Contrasuppression seems to be induced at doses of antigen optimal for immune responses ([6, 7], T. Lehner, personal communication). Certain antigen-presenting cells, such as Langerhans cells, dendritic cells, and peritoneal exudate macrophages induced by complete (but not incomplete) Freund's adjuvant, preferentially activate the cells of this circuit [8, 9]. Other factors involved in activation have been reviewed elsewhere [10, 11].

Following activation, several events have been elucidated. A circuit of T cell interactions has been defined on the basis of surface characteristics of the communicating T cells and the nature of their functional molecular products.

The first subset that has been characterized as follwing activation is an I-J$^+$, Ly-2 T cell which functions to induce contrasuppression [12]. The I-J determinants detected on the cells and molecules of the contrasuppressor circuit are serologically distinct from those expressed by cells and molecules of the "feedback" suppressor circuit [13]. The product of the inducer cell is a molecule(s) which bears an I-J subregion encoded product and can be absorbed on the immunizing antigen (or closely related antigens). The cross-reactive nature of this antigen recognition distinguishes this molecule from suppressor factors [14], and is potentially extremely important. This will be discussed further in the next section. The contrasuppressor inducer factor must interact with a contrasuppressor transducer cell in order to have its effects. This transducer cell is an I-J$^+$, Ly-1, 2 T cell [12, 14]. The evidence at hand suggests that this interaction is restricted by genes linked to the V region of the Ig locus.

The effector cell of the contrasuppressor circuit is an I-J$^+$, Ly-1 T cell which can be positively selected by adherence to the *Vicia villosa* lectin [15], which distinguishes it from helper cells. Further, its activity can be blocked by the presence of *N*-acetyl-D-galactosamine [16]. This cell functions to render helper T cells (and probably other cells of the immune system) resistant to suppressor cell signals [15].

Further, this cell has the ability to block tolerogenic signals in vivo (allowing immunity to become manifest) [17].

Contrasuppression has been implicated in the generation and transfer of contact sensitivity [17–19], resistance to malaria infections ([10], R. Mogil, personal communication), and development of the hyperimmune state [20, 21]. We consider next the possibility that this activity functions in the immune response to cancer.

B. Evidence for Contrasuppression in Tumor Immunity

While contrasuppressor cells have not been used to modulate directly the immune response in cancer, they have been implicated in a number of systems. In this section we will discuss the involvement of contrasuppression in tumor immunity.

Hamaoka et al. [22] described an immunization protocol which produced hapten-reactive T-lymphocytes in the "absence" of suppressor cells. Recently, Rozyka et al. (manuscript in preparation) have demostrated the production of a potent contrasuppressor factor from cells that were primed using Hamaoka's immunization protocol. Hamaoka et al. [23] have further demonstrated that primed animals can produce effective immunity to haptenated tumor cells. Thus, it is likely that activation of contrasuppression to interfere with suppressor cell activity is responsible for the enhanced immune response against the haptenated tumor cells. This is further supported by the observation that the immunity, with time, became cross reactive, such that after priming resistance could be demonstrated for the same tumor cells without hapten [23]. This may be a reflection of the cross-reactive nature of the contrasuppressor inducer cell discussed above [14], that is the reactions against the hapten-modified tumor-associated antigens raised contrasuppressor cells that protected the cells reacting to "unmodified" antigen from host suppressor mechanisms.

Contrasuppression may be implicated in natural resistance to AKR leukemia virus. Mureullo and McDevitt [24] demonstrated

that the transfer of resistance to oncogenesis was dependent upon an I-J$^+$, Ly-1 T cell, a cell with a "contrasuppressive phenotype". In addition, resistant animals could be rendered sensitive by injecting anti-Ly-1 or *anti-I-J antisera* in vivo. Since the effector cell of contrasuppression is an I-J$^+$, Ly-1 T cell [15], removal of this cell could account for the above observations.

Cells which interfere with suppressor cell function were implicated in genetic resistance to Friend leukemia virus (FLV) by Kumar and Bennett [25]. Susceptibility to leukemogenesis correlates with susceptibility to immunodepression by FLV [26]. Susceptibility to immunodepression was further correlated with ability to induce suppressor cells in vitro with FLV [27]. Resistance to suppressor cell induction by FLV was shown to be effected by a marrow dependent cell ("M cell"). Removal of the M cell allowed induction of suppressor cells in resistant strains [25]. (Contrasuppressor cells have been identified in bone marrow and shown to be involved in regulation of hematopoeisis [28].) Kumar and Bennett went on to describe a "suppressor interfering cell" in the FLV system [29]. This will be considered in more detail in the next section. These observations support a role for contrasuppression in control of immunity to leukemia.

Antibodies to certain tumor antigens may react with immunoregulatory cells [30]. Antisera against the Meth A fibrosarcoma raised in Fl animals, but not syngeneic homozygous animals, have been shown to disrupt contrasuppressor activity. Production of these disruptive antibodies correlates with an increased incidence of metastasis in Fl animals over the parental strain [31].

While it remains to be proven that contrasuppressor cells are needed for optimum tumor immunity, the evidence is compelling that this investigative avenue is worth following. In the next section we will consider the activation of this circuit and hypothesize a role for antigen presentation in the context of class I (rather than class II) antigens.

C. Class I Antigens in Contrasuppression and Tumor Immunity

In recent years it has become dogma that helper T cells recognize antigen in the context of class II surface antigens for the initiation of immune responses. Class I antigens are generally viewed as targets for effector cell (CTL) function, such as in T cell killing of transformed or virally infected targets. With few exceptions Ir gene effects mapping to class I loci mediate responses to viral antigens [32] or minor histocompatibility antigens [33]. It is becoming increasing clear, however, that class I antigen presentation in cell-mediated immunity may well involve activation of immunoregulatory subsets. Such regulation has implications for humoral immunity as well.

Using H-2D region mutants, Stukart et al. [34] demonstrated a role for the H-2D halotype in regulating responses to Moloney leukemia virus, even when the effector cells were directed only at virus associated with K-end antigens. H-2D region control of immune responses has also been observed for radiation leukemia virus-induced tumorigenesis [35], Friend virus-induced splenomegaly [36], T-lymphocyte proliferative autoimmune responses to thyroglobulin [37], antibody levels and cellular infiltration in autoimmune thyroiditis [38], and ability to induce suppression for contact sensitivity with DNFB [39]. Antibody responses to equine myoglobin are regulated by complementing genes in H-2D and I-A [40].

Murine resistance to malaria may depend upon activation of contrasuppressor cells to overcome suppression ([10], R. Mogil, personal communication). Vaccination against fatal malaria infection is dependent upon the transfer of infected reticulocytes which display elevated levels of class I antigens [41]. Resistance, however, does not necessarily depend upon the parasite residing within reticulocytes, as immunization with the organism in reticulocytes leads to protective immunity against a fatal strain that proliferates only in mature red blood cells. This indicates that malarial parasites in reticulocytes are not simply better targets of effector cell activity.

Class I antigens have been shown to be important in induction of immunity in several tumor systems. SJL reticulosarcoma lines bearing H-2D antigens are capable of inducing immunity to lines which lack H-2D [42]. Examination of progressor and regressor lines of a UV-induced sarcoma revealed an anitgenic difference mapping to the H-2D region of the MHC. Again, like the Hamaoka story and the immunity to malaria, the regressor line was found to be capable of inducing immunity to the progressor line which lacks the H-2D linked antigen [43].

In the FLV system, Kumar and Bennett [29] examined an FLV-induced "suppressor interfering cell" which was activated in vitro by genetic mismatch of this cell with its target. (The H-2 haplotype of the FLV-induced suppressor cell was irrelevant.) This allogeneic activation was mapped to H-2D [44].

Recently, a system has been developed to analyze the activaton of contrasuppression by antigen-presenting cell subsets in vitro. Preliminary results suggest that this antigen-specific activation can be blocked by anti-class I (especially H-2D) but not by anti-class II antibodies (in preparation).

In light of the above observations, we propose that antigen presentation in the context of class I antigens, especially H-2D, may be important in initiation of contrasuppression. It may be relevant that dendritic cells, which can activate contrasuppression which leads to *dominant* immunity in vitro [9], are high in H-2D antigen expression [45].

If so, then a strategy for optimal tumor immunity may be elevation of class I antigen expression on the tumor cells to activate contrasuppression and allow dominant immune responsiveness over tumor-induced suppression. Experiments are in progress to test this notion.

D. Flip Side: Contrasuppression in Enhancement of Lymphoid Tumor Development

It is well established that persistent activation of target cells by their hormones can result in transformation and carcinogenesis. Regulatory factors are essentially the hormones of the immune system, and we can propose that persistent activation of their targets can result in neoplasia. Signals which inhibit activation, such as suppressor cell factors, might then serve to prevent lymphoid transformation, whereas activities like contrasuppression might, in some instances, enhance lymphoid tumorogenesis.

For example, Houghton et al. [46] have described a situation in which antigenic hyperimmunization causes the appearance of tumors of cells of the immune system. The fact that several B-cell lymphomas produced in this way react with the immunizing antigen suggests direct involvement of the hyperimmunization protocol. Hyperimmunized animals have been shown to possess a potent antigen-specific contrasuppressive activity [20, 21].

As mentioned above, malaria infections in mice produce a potent contrasuppression coincident with recovery. Such infections can enhance oncogenesis by virus [47]. Whether there is any correlation of these effects is unknown, but suggests an exciting possibility. People infectd with Epstein-Barr virus (EBV) exhibit potent suppressor T-cell activity [4], and such cells have been shown to be capable of inhibiting EBV transformation in vitro [5]. Chronic infection with malaria, however, might induce a general contrasuppression which would interfere with this beneficial immunosuppression to allow expansion of the virus-transformed cells. This is a possible rationale for the association of EBV-induced lymphomas in malarial regions [48].

The MRL mouse is a murine model of systemic lupus erythematosis and lymphoproliferation in which autoimmunity proceeds in the face of general suppression [49]. These animals have been shown to be resistant to tolerance induction [50] and suppressor cell signals [51], probably as a result of excessive contrasuppressor activity [51]. The proliferating cells in these animals have a controlled neoplastic tendency, as suggested by the spontaneous appearance of transformed, tumorgenic lines when these cells are cloned (C. Reinisch, personal communication). An understanding of the role of immunoregulatory T cells in the control of such lymphoid tumors will great-

ly increase our knowledge of lymphocyte regulation and the regulation of transformed cells in general.

E. Conclusion

In this brief discussion, we have outlined our argument that contrasuppression might play an important role in the immune response to cancer. While antigen load often induces active suppression to most tumors, induction of contrasuppression early in the response might allow protective immunity to become dominant. There is suggestive evidence that contrasuppression can be initiated by presentation of antigen in the context of class I antigens, in which case these will have a profound role in determining the outcome (positive versus negative immunity) of a tumor challenge.

Many tumors can potentially be controlled by immune responses *against* the tumor. Certain tumors, in addition, might be affected by the regulatory molecules of the immune system themselves, especially if the tumors are lymphoid in origin. Such tumors might behave anomalously (on the surface), being enhanced by positive influences on immune function and controlled by suppressive signals.

Nevertheless, it is clear that as our understanding of immunoregulation increases, we simultaneously improve our potential for controlling the immune response to cancer and increase our abilites to produce effective therapy.

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Inhibition of the Induction of Contrasuppression by Antisera Against Tumor-Associated Surface Antigens on Methylcholanthrene-induced Sarcomas*

P. M. Flood, A. B. DeLeo, L. J. Old, and R. K. Gershon

A. Introduction

Contrasuppression is an immunoregulatory T-cell activity that protects *Lyt* 1^+, 2^- T-helper cell activity from suppression. This activity involves both an "induction" (afferent) phase, which requires the activation of an *Lyt* 1^+, 2^- effector T cell by cells in the contrasuppressor circuit [6], and an "effector" (efferent) phase, in which the effector cells or cell-free products secreted by these cells render T_H cells resistant to suppression [4]. Recently we discovered an activity in antisera raised against methylcholanthrene-induced sarcomas from Balb/c mice, which blocks T-cell regulatory activity [3]. These antisera block the afferent as well as the efferent phase of suppression to SRBC in vitro, but only in animals which express the same Igh gene polymorphism as Balb/c (Igh^a). We therefore tested whether these antisera could block the afferent and efferent phases of contrasuppression, and whether this activity had any effect on the growth of tumors in those mice.

B. Materials and Methods

The chemically induced sarcomas, and the antisera against them, were prepared according to procedures described by DeLeo et al. [1, 2]. Suppressor T cells were prepared according to the method of Janeway [5]. Contrasuppressor T cells were prepared according to the method of Green [4]. Contrasuppressor factor ($T_{CS}F$) is a cell-free supernatant collected from in vitro generated T_{CS} cells. Generation of primary anti-SRBC cultures and blocking assays with antisera has been described [3]. Assays for metastasis were performed by injecting 10^5 or 5×10^4 Meth A cells into the right footpad of test animals. After 3–4 weeks, lymph nodes were removed and weighed and examined histologically for evidence of tumor cell growth. Animals positive for metastasis were those which showed tumor cell growth in the popliteal lymph nodes of the left leg, as well as both axilary lymph nodes.

C. Results

Antisera effective in blocking the afferent but not the efferent phase of suppression were tested for their abilitiy to block the afferent and efferent phases of contrasuppression (Table 1). Antisera raised in syngeneic Balb/c mice against Meth A (or other MC-induced tumors, data not shown) were ineffective in blocking the activity of either the $T_{CS}F$, which represents the efferent phase of contrasuppression, or the T_{CS} cells, which represents the afferent phase of contrasuppression. However, antisera raised in semisyngeneic $CB6F_1$ or *Igh* congenic C.B20 mice effectively blocked the activity of the Balb/c T_{CS} cells but not the $T_{CS}F$. Likewise, these antisera were very effective in blocking afferent T_{CS} activity in $CB6F_1$ mice, while they were ineffective in blocking T_{CS} activity in *Igh* disparate mice, reiterating the earlier finding on the nature of

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Table 1. Antisera to Meth A raised in Igh^{b+} mice block contrasuppression

Assay cells[a]	Antisera	Anti-SRBC PFC/culture			
		–[b]	T_s	$T_s+T_{cs}F$	T_s+T_{cs}
Balb/c (Igh^a)	–	1600	300	1400	1200
Balb/c (Igh^a)	Balb/c anti-Meth A	1400	400	1400	1400
Balb/c (Igh^a)	$CB6F_1$ anti-Meth A	1800	200	1400	200
Balb/c (Igh^a)	C.B20 anti-Meth A	1500	300	1500	100
$CB6F_1$ (Igh$^{a/b}$)	–	6000	1200	4900	5400
$CB6F_1$ (Igh$^{a/b}$)	Balb/c anti-Meth A	6700	1000	5700	5000
$CB6F_1$ (Igh$^{a/b}$)	$CB6F_1$ anti-Meth A	8000	1500	4600	2400
$CB6F_1$ (Igh$^{a/b}$)	C.B20 anti-Meth A	7200	1100	4100	2800
C.B20 (Igh^b)	–	3100	1100	2300	2700
C.B20 (Igh^b)	Balb/c anti-Meth A	2900	900	2100	2600
C.B20 (Igh^b)	$CB6F_1$ anti-Meth A	3000	1100	2400	2400
C.B20 (Igh^b)	C.B20 anti-Meth A	3500	1000	2200	2800

[a] 10^7 unprimed spleen cells were stimulated in primary anti-SRBC cultures for 5 days. The Igh haplotypes of the spleen cells are given in parentheses

[b] Antisera were added at a final concentration of 1% on day 0 of culture. Cultures marked with – indicate cultures of spleen cells only; T_s indicates cultures of spleen cells $+2\times10^5$ syngeneic T suppressor cells: $T_s+T_{cs}F$ indicates cultures of spleen cells, syngeneic suppressor cells at 2×10^5, and T contrasuppressor factor added at a final dilution of 10% on day 0 of culture; and T_s+T_{cs} indicates cultures of spleen cells, T suppressor cells at 2×10^5, and syngeneic T contrasuppressor cells at 2×10^5

the Meth A antigen [3]. When antisera was absorbed with tumor cells passed in either Balb/c or $CB6F_1$ mice, only F_1 passed tumor cells absorbed the activity (Table 2), suggesting a higher density of relevant antigen on cells passaged in F_1 mice. When Balb/c, $CB6F_1$, or C.B20 tumor-bearing mice were assayed for metastasis, in repeated experiments less than 15% of Balb/c or C.B20 mice had lymph node metastasis, while greater than 93% of $CB6F_1$ mice developed metastasis after injection of Meth A cells.

D. Discussion

An additional activity, the blocking of contrasuppression, has been found in antisera

Table 2. Tumors passed in $CB6F_1$ but not Balb/c absorb blocking activity

Assay cells[a]	Absorbing cells[b] passed in:	Anti-SRBC PFC/culture[c]		
		–	T_s	T_s+T_{cs}
Balb/c	No sera added	1600	300	1200
Balb/c	Sera not absorbed	1600	300	500
Balb/c	Balb/c	1700	300	700
Balb/c	$CB6F_1$	1500	200	1300
$CB6F_1$	No sera added	7000	2000	5700
$CB6F_1$	Sera not absorbed	8000	2100	2800
$CB6F_1$	Balb/c	7400	1800	3100
$CB6F_1$	$CB6F_1$	7400	2100	5900

[a] See footnote [a], Table 1

[b] Meth A cells passed in either Balb/c or $CB6F_1$ mice were used to do a double absorbtion of the antisera as described [1]

[c] See footnote [b], Table 1

against MC-induced tumors. Experiments with F_1 and *Igh* congenic mice indicate that effective antisera can only be generated in mice containing *Igh* disparate genes, while activity is only directed against cells expressing the Igh^a gene locus. This brings up the apparent dichotomy that F_1 mice generate autoantibody to their own *Igh*-linked gene products. However, tumors passaged in F_1 animals express the relevant antigen in a much higher surface density than does the parental strain. This "adaptive differentiation" process may explain the difference in tumorgenicity between F_1 and Balb/c mice, as measured by metastasis. The intriguing possibility exists that F_1 mice produce autoantibodies that block the generation of their own contrasuppressor cells, and that these contrasuppressor cells are important in controlling tumor metastasis. It also suggests that while many tumor cells escape immune destruction by generating suppressor T cells to depress immune responses, malignant cells may also escape by "encouraging" immunity, e.g., generating antigens which mimic normal cellular interaction structures and thereby blocking important cellular communication mechanisms needed to generate effective antitumor immunity.

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Dissection of a Unique Tumor-Specific Transplantation Antigen into Multiple Unique Independent Epitopes using Syngeneic T-Cell Lines*

R. D. Wortzel, C. Philipps, J. L. Urban, and H. Schreiber

In our past and present studies, we have analyzed the tumor-specific antigens and T-cell clonotypes that are involved in syngeneic tumor rejection. We expect that such information will be very useful for learning to manipulate tumor-specific immunity. As our tumor model, we have studied the murine ultraviolet-light (UV) induced fibrosarcoma 1591-RE and characterized its tumor-specific transplantation antigen using, as immunologic probes, syngeneic tumor-specific T-cell lines generated from animals that had rejected the tumor. This 1591 tumor, like many other UV-induced tumors, is very immunogenic and is routinely rejected by normal syngeneic mice [1]. We have previously shown that this resistance of the normal mice is dependent upon idiotypically restricted tumor-specific T cells [2, 3] which are specific for a unique 1591-speific tumor antigen. On rare occasions, normal mice develop progressively growing 1591 tumors, and these tumors (1591-PRO) are always heritable variants that have lost a unique regressor tumor-specific transplantation antigen [4]. (This loss has been determined by the resistance of the progressor variants to cytolytic T cells raised against the regressor tumor.)

In more recent studies, we have generated a syngeneic cloned cytolytic T-cell line that demonstrated the regressor-specific reactivity pattern, and we defined the epitope recognized by this T-cell clone as the "A" epitope [5]. We then used this cytolytic anti-A T-cell clone to select in vitro for tumor variants missing the A epitope in an attempt to dissect the tumor-specific transplantation antigen. The A^- variants were found in the 1591-RE population at a frequency of about 1 in 10^4 tumor cells, and these variants were not only resistant to the anti-A T-cell clone but also to cytoltic T cells from mixed lymphocyte-tumor cell bulk cultures (MLTC) responding to 1591-RE tumor cells. This suggested that the A epitope was present on a major tumor-specific transplantation antigen recognized by the host response, and that this antigen had been lost due to selection in vitro with the anti-A T-cell clone.

Similar to the in vivo derived variants, this A^- variant also grew progressively in normal mice at high doses and smaller doses of these variants could be rejected by normal mice. Interestingly, these mice which rejected A^- cells generated an immune response that lysed the A^- variants, and in agreement with our earlier studies [6], these anti-A^- bulk MLTC cells also lysed the original 1591-RE regressor tumor. The response was 1591-specific since no other UV-induced fibrosarcoma lines tested were lysed. We then derived T-cell lines from these MLTC cells, and, despite cloning, these T-cell lines retained a dual specificity pattern. This clearly indicated that the 1591-RE, the 1591-A^- variants, and the host selected 1591-PRO variant tumor cells all expressed one common tumor-specific epitope which was probably different from the A epitope. This epitope was, therefore, named the B epitope.

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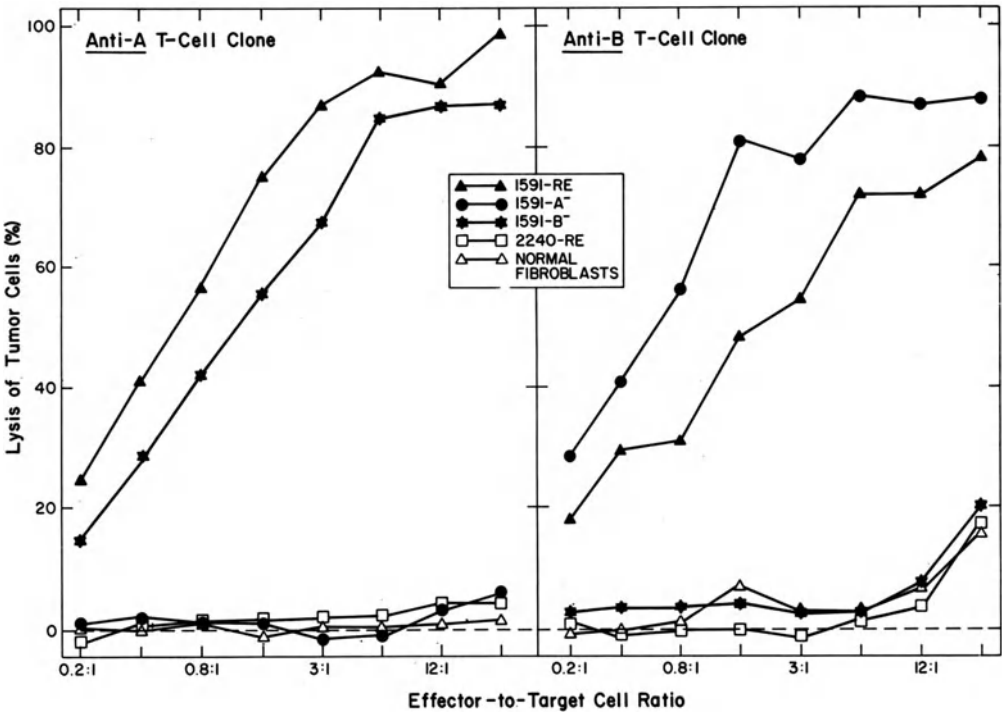

Fig. 1. Selective resistance of A^- and B^- 1591 tumor cell variants to either anti-A (*left*) or anti-B (*right*) T-cell clones as measured in a ^{51}Cr-release assay. For details see [5].

To further define the interrelationship of the two unique 1591-specific epitopes on the parental 1591 tumor, we determined next whether the loss of the A or the B epitope was independent or linked. Thus, we selected for 1591 tumor variants which were resistant to the anti-B T-cell line. We found that the B^- variants retained the A epitope while the A^- variants had retained the B epitope (Fig. 1). A similar "flip-flop" pattern was found upon analysis of the antigen dependence of the two T-cell lines (Fig. 2). The growth of both T-cell lines was stimulated by the 1591-RE cells, whereas 1591-A^- variant cells and 1591-B^- variant cells stimulated the T-cell line with the relevant specificity.

Six more variants derived by the same general protocol also showed independent loss of the A and B epitopes. This proves that the A and B epitopes are different and are not closely linked. The results are also inconsistent with the idea that the anti-B cell line is a high affinity clone for the A epitope. In other studies, which we will not elaborate on, we have found that there is still a third also unique 1591-specific epitope which we call the C epitope. Again, this epitope was defined by a syngeneic T-cell line and could be lost independently from the A and B epitopes. Obviously, if all these epitopes were expressed on one and the same antigenic molecule, we would have probably observed at least in some variants a simultaneous loss of more than one epitope after selection with a single cytolytic T-cell clone. Therefore, it appears more likely that these epitopes reside on different antigenic molecules.

At present, we can only speculate on the origins of multiple unique tumor-specific antigens suggested by the results of our studies. However, it is highly unlikely that they represent normal C3H histocompatibility antigens recognized by C3H mice due to "genetic drift" of the responder's histocompatibility antigens. If such genetic drift had occurred in the mice since the 1591 tumor had been derived, one would expect that the isolated T-cell probes would also react with the other UV control tumors which had been isolated at the same time,

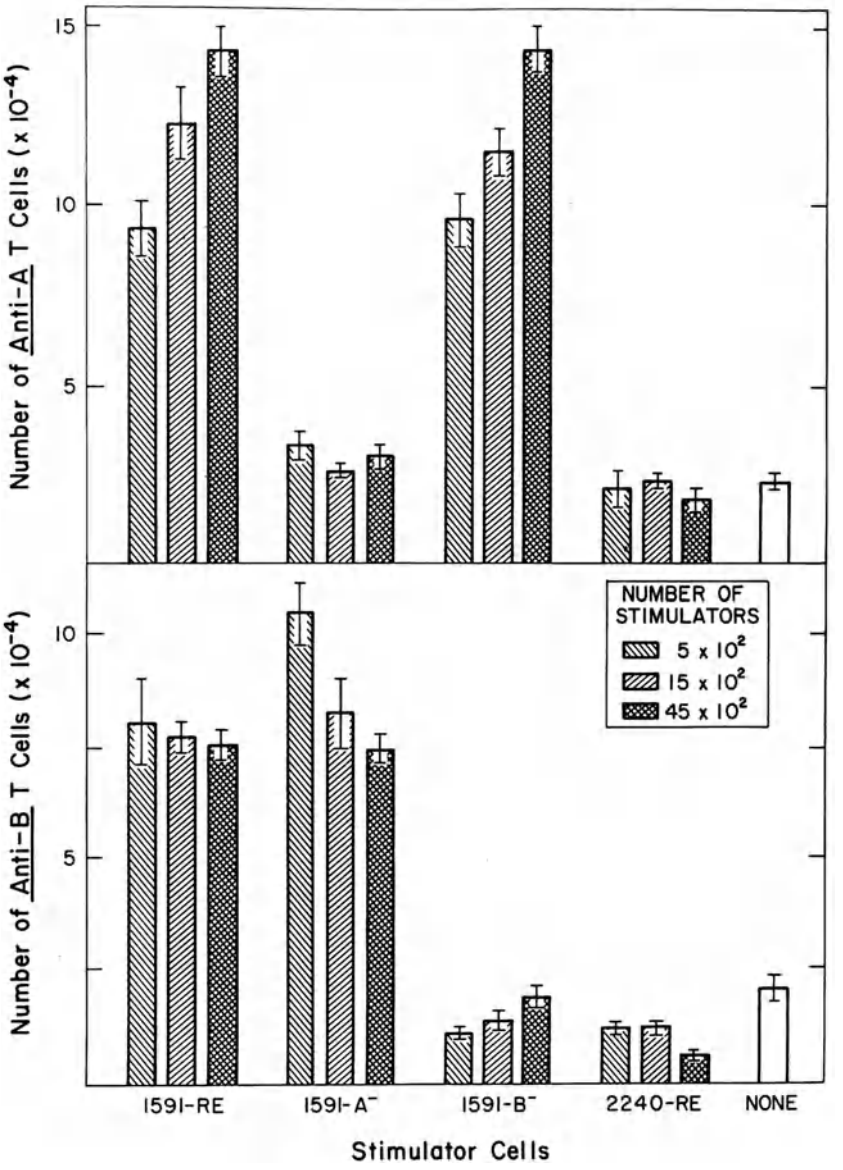

Fig. 2. Selective stimulation of anti-A (*upper*) and anti-B (*lower*) T-cell clones with either A^- or B^- 1591 tumor cell variants as measured by relative increase in T-cell numbers during a 7-day coculture with stimulator cells. For details see [5]

in the same experiment, and in the same stock of mice.

It is important to mention that despite this multiplicity of tumor-specific epitopes on the 1591 tumor cell, previous experiments have shown a clear hierarchy in the recognition of these epitopes [7]. The immune response of normal mice was always found to be directed to the A epitope, which was immunodominant over the immunorecessive B epitope; the B epitope was only recognized by the host once the A epitope had been lost from the 1591 cell. This hierarchy is in agreement with other earlier studies showing idiotypic restriction of the T-cell response to 1591-RE tumor cells. Obviously, the restriction of the immune response to certain immunodominant tu-

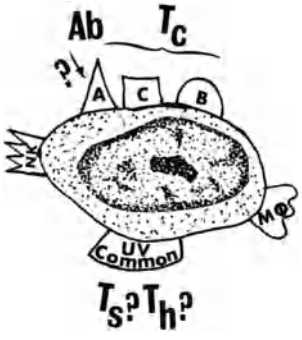

Fig. 3. Hypothetical scheme of the surface antigens on the parental 1591-RE tumor cell. While we consider it likely that the unique A, B, and C epitopes are on separate surface antigens, it is not clear whether the common UV antigen is part of an antigenic moiety common to all unique antigens, or a separate surface antigen. For details on the NK and macrophage-recognized target sites see [6, 7]

mor-specific epitopes on the malignant cell increases the chance of the tumor being able to escape immune destruction, because it only has to undergo one phenotypic change in order to escape immune destruction. On the other hand, it is clinically important that such variant tumors which have escaped the immune defense of the host probably retain other tumor-specific epitopes that can still act as targets for immunotherapy. Figure 3 shows a hypothetical schematic drawing of the antigenic surface makeup of the parental 1591- RE tumor cell. We have not discussed the data consistent with the existence of a different UV antigen which is common to all UV tumors. This antigen, which is included for completeness in Fig. 3, appears to be primarily recognized by regulatory antitumor immunity, such as that providing help or suppression [8, 9]. An analogous general functional separation of antigenic recognition appears to occur in allogeneic responses to so-called class I and class II histocompatibility antigens [10]. So far, we have not yet found evidence that the host can select against the expression of this common UV antigen. While, on the other hand, the multiple, unique tumor-specific epitopes discussed above appear to be the targets of cytolytic and possibly other types of immunoselective effector immunity and are sequentially selected against in an order which depends upon the hierarchy in the tumor-specific immune response.

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Detection of Antigen-(AKR MuLV gp70)-Specific Circulating Immune Complexes (CIC) in Mice with Lymphomas*

H. Rohmer, H. Schetters, A. Luz, R. Hehlmann, and V. Erfle

A. Introduction

Circulating immune complexes (CIC) probably play an important role in a variety of human and animal neoplastic diseases. CIC may represent the major part of the humoral factors which inhibit cell-mediated reactivity to tumor cells [1]. Furthermore, CIC in tumors seem to be a prognostic factor for the course of the disease [2, 3]. In some reports leukemias and lymphomas in man have been reported to be associated with immune complexes [7, 8, 10, 11] and have been shown to represent an unfavorable prognostic factor [2].

The specificity of the antibody IgG moiety and hence the nature of the complexed antigens in CIC in leukemias and lymphomas is unknown. Recently we showed the presence of CIC with retroviral antigens in some patients with CML blast crisis [5]. Retroviruses as possible causative factors of leukemias and lymphomas in men and animals are therefore probably involved in the formation of CIC. To resolve this question, mice with lymphomas of different origin were examined as model systems for the presence of CIC and especially for the presence of CIC containing retroviral envelope proteins.

B. Methods

CIC with undefined antigen were detected by a C1q-binding test according to Wehler et al. [12], using the ELISA technique. CICs with AKR MuLV gp70 were determined by the ELISA technique using a AKR MuLV gp85 rabbit antibody (gift of Dr. G. Hunsmann, Institut für Immunbiologie, Freiburg) as coating serum.

The serum samples were diluted 1 : 100 in assay buffer (PBS, 0.1% Tween 20, 0.5% Trasylol, 0.1 m*M* Thimerosal, 1% BSA), added to the well, and incubated at 4 °C overnight. AKR MuLV gp70 containing immune complexes present in the serum were determined by adding peroxidase-coupled rabbit anti-mouse IgG diluted in assay buffer. *O*-phenylenediamine-2HCl was used as substrate. The absorbance was measured at 450 nm with a Titertek Multiscan or a SEI/Kontron SLT 210. AKR MuLV gp70 and antibodies against AKR MuLV gp70 were detected with the ELISA technique [4, 9]. All mouse strains were from the breeding colony of the Institute of Biology of the GSF.

C. Results

Three different types of murine lymphomas have been examined for the presence of CIC, the spontaneous T-cell lymphomas of AKR mice, the X-ray induced T-cell lymphomas of C57Bl/6 mice, and the spontaneous late B-type lymphomas of low leukemia mouse strains (BALB/c, X/Gf, CBA). Age-matched healthy animals served as controls (AKR: 5–8 months; C57Bl/6: 5–8 months; BALB/c, NMRI, X/Gf, CBA: older than 18 months).

* In association with Euratom (Contract No. BIO-D-366-81-D)

I. Spontaneous AKR T-Cell Lymphoma

The spontaneous T-cell lymphomas of AKR mice have been found to develop during months 7–10. AKR mice with lymphomas (with the exception of two animals) did not show elevated CIC or AKR MuLV gp70 containing CIC in comparison to age-matched AKR mice or control mice of other strains (Figs. 1, 2). The AKR mice harboured a remarkable amount of AKR MuLV gp70 of 6–14μg/ml serum

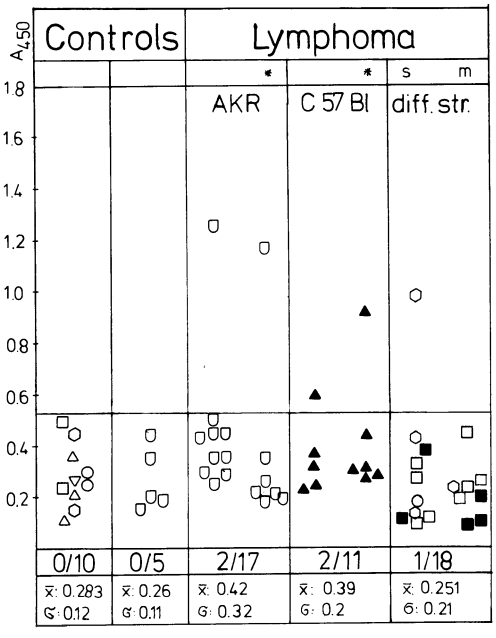

Fig. 1. C1q-binding circulating immune complexes in the sera of lymphoma-bearing mice. *Symbols:* ⌓ AKR, □ BALB/c, ◇ X/Gf, ○ CBA, △ C57Bl/6, ▽ NMRI. *Closed symbols* represent animals which had been irradiated with X-rays (column 4) or ^{227}Th (column 5). * The animals in these columns had been pretreated with alkylphospholipid (ALP); *s*, animals with lymphoma only; *m*, animals with an additional tumor to the lymphoma; the *horizontal lines* represent the mean value plus 2σ of the respective control animals; *column 1*, healthy animals of different mouse strains; *column 2*, healthy AKR mice; *column 3*, AKR mice with lymphoma; *column 4*, C57Bl/6 mice with lymphoma; *column 5*, mice of different strains with lymphoma. At the *bottom of the columns* the number of positive mice to the number of mice examined is indicated together with the mean value of the group plus standard deviation (σ)

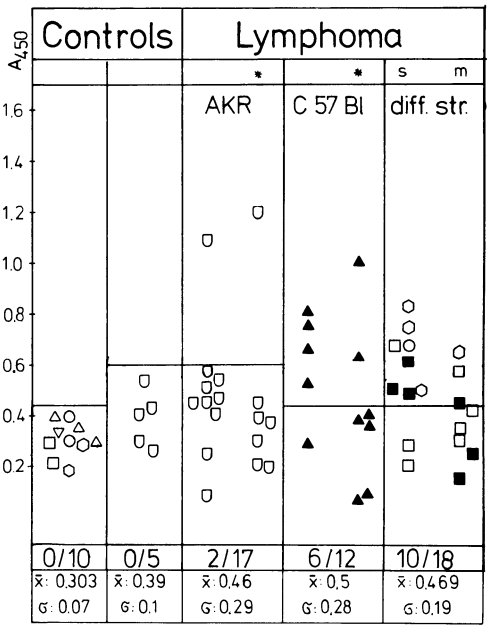

Fig. 2. AKR MuLV gp70 specific circulating immune complexes in the sera of lymphoma-bearing mice. For symbols see Fig. 1

(mean value 10 μg/ml). This was seen in lymphoma animals and in control mice (Fig. 3). Except for one mouse with lymphoma no AKR MuLV gp70 specific antibodies were detected in either the lymphoma group or the controls. Also no differences were noticed in one of the parameters between mice treated with alkyllysophospholipid (ALP) as an antitumor drug and their untreated counterparts.

II. X-Ray Induced C57Bl/6 T-Cell Lymphoma

The irradiated animals developed lymphomas 7–8 months after X-ray treatment. Only 2 out of 11 tumor-bearing mice had elevated values in the C1q-test for CIC. In contrast half of the mice (6 out of 12) with X-ray induced lymphomas showed increased levels of AKR MuLV gp70 specific CIC (Wilcoxon test significant against controls; Fig. 2). But there was a difference between lymphoma mice which had been pretreated with ALP and untreated mice. Less ALP-treated mice (two out of seven) exhibited specific CIC above normal than

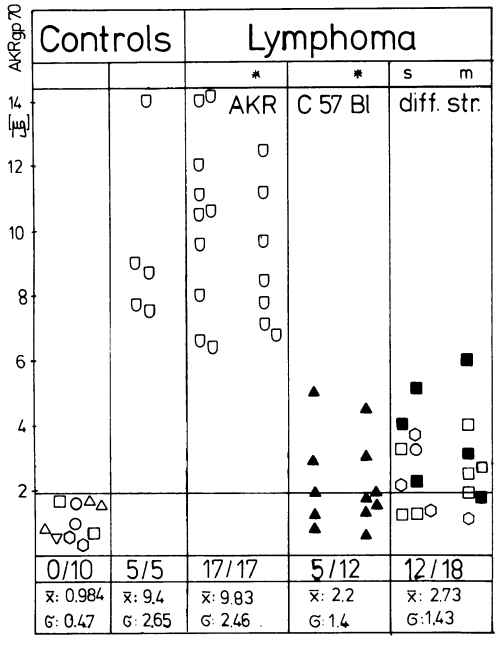

Fig. 3. AKR MuLV gp70 in the sera of lymphoma-bearing mice. For symbols see Fig. 1

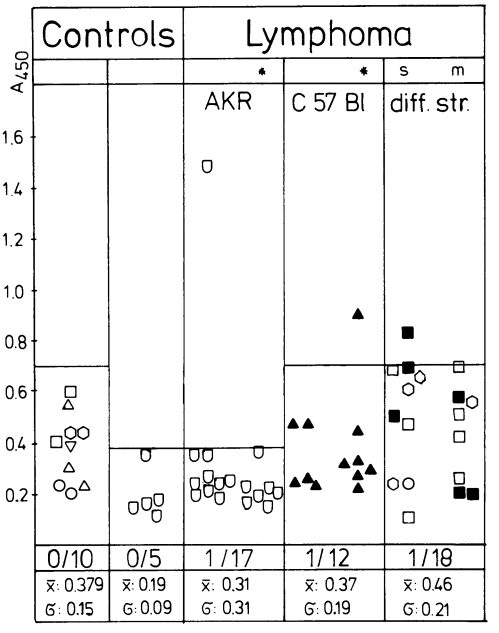

Fig. 4. Antibodies against AKR MuLV gp70 in the sera of lymphoma-bearing mice. For symbols see Fig. 1

untreated controls (four out of five). Also 5 out of 12 lymphoma animals had AKR MuLV gp70 serum levels above normal (2–6 µg/ml) (see Fig. 3). Only one mouse (ALP treated) with elevated AKR MuLV gp70 antibodies was detected. ALP-treated and untreated mice had an almost similar pattern concerning viral protein and viral antibodies.

III. Spontaneous Late B-Type Lymphoma

Spontaneous B-Type lymphomas (according to the classification of T. Dunn) appeared in the low leukemia mouse strains BALB/c, X/Gf, and CBA between the 20th and the 25th months of life. Some of the animals were suffering from a second malignancy (predominantly alveolar lung carcinomas) in addition to the lymphoma (see Figs. 1–4; column 5, m). A portion of the mice had been injected with ^{227}Th for osteosarcoma induction, but had not developed osteosarcoma.

Elevated C1q-binding CIC were generally not detected in this group of mice (1/18). In contrast, a significant number of animals (10 out of 18) were found to harbour AKR MuLV gp70 containing CIC above the background of healthy age-matched mice (Wilcoxon test significant). The same result was obtained for AKR MuLV gp70 antigen bodies (Figs. 2, 3). Twelve out of 18 lymphomatous mice had increased gp70 serum levels (2–6 µg/ml). The viral antibody titers remained in the normal range (see Fig. 4; column 5). No major differences were found between animals with only lymphoma (s) or with an additional second tumor (m).

D. Discussion

Circulating immune complexes detectable by a C1q-binding test can only rarely be observed in lymphoma-bearing mice. This is in some contrast to the situation with leukemias and lymphomas in men where about 30%–40% of the patients have a significant increase of C1q-binding CIC [2]. The possibility of a minor sensitivity of the test system used in our experiments could be ruled out by the detection of high

amounts of CIC in the serum of NZB mice known to develop an autoimmune disease accompanied by CIC formation (data not shown).

On the other hand antigen-specific (AKR MuLV gp70) CIC were present in the sera of C57Bl/6 mice with X-ray induced T-cell lymphomas and in the sera of low leukemia mice with late spontaneous lymphomas (T. Dunn type B). These CIC are obviously below the detection level of the C1q-binding test used. The presence of AKR MuLV gp70 specific CIC is accompanied by elevated AKR MuLV gp70 in the serum and normal antibody titer against AKR virus. This pattern clearly separates lymphoma animals from healthy individuals. Whether this situation indicates an antigen excess or antigen-antibody complexes with free antibody binding sites is unknown.

A different pattern has been observed from that described above in lymphoma-bearing AKR mice. Despite high serum levels of AKR MuLV gp70 no increased antibodies or CIC against this antigen were detected. The same situation was also found in healthy AKR mice. This describes a total immunotolerance of AKR mice against this endogenous viral glycoprotein. The anti-AKR MuLV gp85 antibody used in these experiments had a broad reactivity against ecotropic (Friend MuLV) and xenotropic (BALB virus: 2) murine retroviruses (G. Hunsmann, personal communication). It should therefore be reasonable to assume that all MuLV glycoprotein specificities which have been described in preleukemic and leukemic AKR mice [6] should have been detected.

We have no information on the prognostic value of the antigen-specific CIC in mice because the animals had been killed at the time of tumor detection. Our recent finding of a shorter survival time of those patients in CML blast crisis who had SiSV gp70 specific CIC or antigens in comparison to negative patients indicates such a possibility. This observation is in perfect accordance to survival data of patients in CML blast crisis with and without C1q-binding CIC [2]. The observation of CIC specific for retroviral antigens in murine and human leukemias and lymphomas suggests that retrovirus antigen-specific CIC in addition to their possible contribution to the course of the disease might be of prognostic value in these malignancies.

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We thank Dr. G. Hunsmann for providing us with the AKR MuLV gp70 antibody.

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Immunological Characterization of the Natural Antibodies to Human T-Cell Leukemia Virus in Human Sera

M. G. Sarngadharan, J. Schuepbach, V. S. Kalyanaraman, M. Robert-Guroff, S. Oroszlan, and R. C. Gallo

Human T-cell leukemia virus (HTLV) is a unique, exogenously acquired human type C retrovirus. Its isolation, characterization, and evidence for its infectious transmission are presented elsewhere in this volume. Here we will discuss some of the biochemical and immunological properties of HTLV *gag* gene products, and summarize the main features of the natural antibodies to these proteins present in some human sera.

Like all the mammalian retroviruses, HTLV contains several small molecular weight proteins such as p19, p12, p24, and p15. These proteins appear to be homologous to the *gag* proteins p15, p12, p30, and p10 respectively of the prototype mouse type C viruses. We have purified p19, p24, and p15 of HTLV to homogeneity ([3]; Schuepbach J, Kalyanaraman VS, Sarngadharan MG, Blattner WA, Gallo RC, to be published). P19 is believed to be the 5′ terminal *gag* protein of HTLV and, like its homologue in murine viruses, appears to be blocked at its 5′ terminus (Oroszlan, unpublished observations). Extensive amino acid sequence analyses have been done on p24 and p15 ([6]; Oroszlan et al., unpublished). While it is quite clear that HTLV and bovine leukemia virus (BLV) are very different viruses, e.g., lack of immunological cross reactivities and little nucleic acid sequence homology, when the amino acid sequences of HTLV proteins were compared with the sequences of the corresponding *gag* proteins of other mammalian retroviruses, there were significant similarities only with proteins of BLV. Thus, between the amino terminal first 25 residues of BLV p24 and HTLV p24, there was correspondence among nine amino acids. Similar correspondence was observed up to the first 150 residues for which sequence data are available. This has been determined to be a statistically significant correlation, one that cannot be a result of chance alone. Similarly the amino acid sequence of HTLV p15 shares extensive homology with the BLV p12. This homology also includes a conservation of the nucleic acid binding domain characterized by the repeated cystine at positions n, n+3, and n+13 and a histidine at n+8, tryptophan at n+9, and aspartic acid at n+12 (Copeland TD, Morgan MA, Oroszlan S, to be published). These data strongly suggest that these homologous proteins of HTLV and BLV may have evolved from common ancestral molecules in some distant past. The extent of amino acid sequence homology described above also underscores the substantial *dissimilarities* between the two viruses and, in fact, the uniqueness of HTLV among mammalian retroviruses. As predicted, and as noted above, we have observed no immunological cross reactivity between HTLV and any other retrovirus, including BLV in conventional radioimmunoassays in either homologous or several widely cross-reactive heterologous systems ([3, 8]; Schuepbach J, Kalyanaraman VS, Sarngadharan MG, Blattner WA, Gallo RC, to be published).

A major finding that helped in assessing the extent of HTLV spread and its relevance to leukemias and lymphomas was that the initial patients whose cultured cells expressed the virus also had specific antibodies in their sera reactive against the protein components of the virus [4, 7]. It was expedient, therefore, to screen sera of pa-

tients for antibodies to HTLV, rather than attempting to isolate virus from each case. Four different methods were used in this large scale screening. (1) Immunofluorescence on HTLV-producing cells upon incubation with the test serum and an appropriate FITC-antibody conjugate [9]; (2) A solid-phase immunoassay using whole disrupted HTLV [7]; (3) A competitive binding assay using a monoclonal antibody to HTLV p19 and disrupted HTLV [10]; and (4) a specific radioimmunoassay using homogeneous core proteins ([4, 5]; Schuepbach J, Kalyanaraman, Sarngadharan MG, Blattner WA, Gallo RC, to be published). Detailed discussion here will be limited to specific immunoprecipitation studies using purified proteins.

Representative patterns of immunoprecipitation curves for ^{125}I-labeled HTLV proteins obtained with some antibody-positive human sera are shown in Fig. 1. The sera are from some Caribbean patients with T-cell lymphosarcoma cell leukemia (T-LCL) [1], some of their relatives, and some clinically normal Caribbean individuals not known to be related to any of the leukemia patients. The results indicate that all the HTLV *gag* proteins tested (p24, p19, p15) are precipitated by these sera. In all cases, the precipitation has been found to be highly specific. Irrespective of the labeled antigen used, the only competition observed is with either HTLV extracts or extracts of a cell line producing HTLV. None of a large number of other mammalian retroviruses including BLV or cultured normal human T cells showed any effect on this precipitation (Fig. 2). Specificity was also observed within HTLV to the particu-

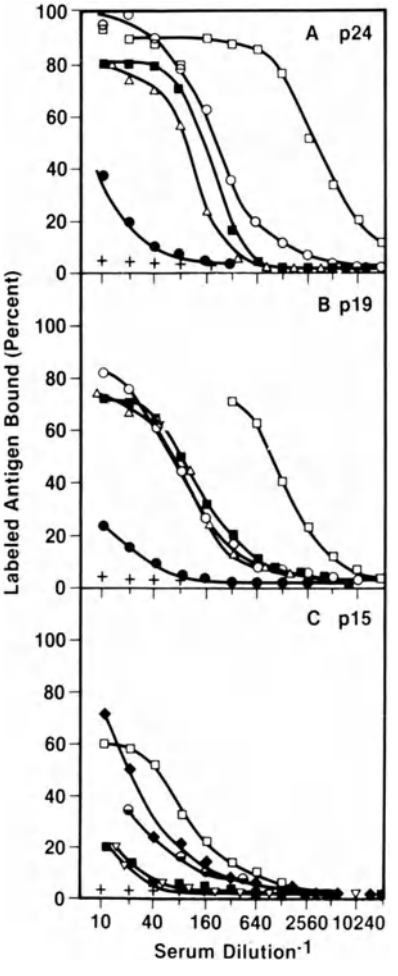

Fig. 1A–C. Representative radioimmune precipitations of purified and labeled HTLV proteins p24 **A**, p19 **B**, and p15 **C** by serum samples of TLCL patients (○, 23.1; ●, 24.1; △, F0706; +, F1123), their family members (■, F0781), and healthy individuals from the Caribbean (□, #81-001; ▽, C-579; ◆, C-581; ◒, C-585). Serial dilutions of serum were incubated with 8,000–10,000 cpm of the lapeled protein. A 30-fold excess of goat anti-human IgG antibody was added, and the percentage of labeled antigen bound in the precipitation was determined

lar protein being precipitated. Thus, when a mixture of ^{125}I-labeled p24, p19, and p15 was immunoprecipitated and the immune complex analyzed by polyacrylamide gel electrophoresis in the presence of SDS, radioactive peaks corresponding to all three proteins were observed (Fig. 3A). Anti-p15 titer was considerably lower than titers against p24 and p19 and, therefore, an aliquot of the serum precipitated less p15 than p24 and p19 (Fig. 3A). Increasing the serum concentration did increase the amount of p15 precipitated (Fig. 3E). When the above precipitation of the protein mixture was repeated in the presence of nonradioactive p24, p19, or p15 in successive experiments, the unlabeled antigens specifically blocked the precipitation of the corresponding radioactive proteins (Fig. 3B–D). Therefore, the serum contained specific and separate antibodies to all these internal viral proteins.

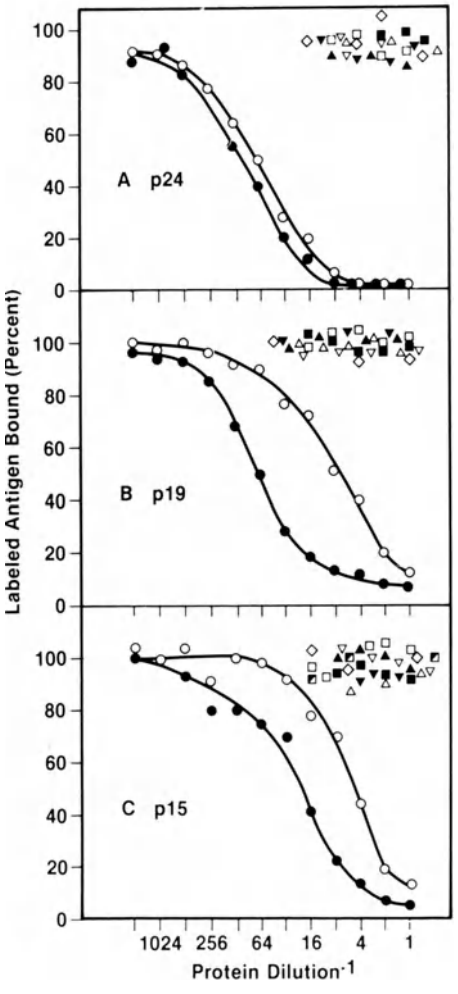

Fig. 2A–C. Viral and cellular competition in the precipitation of HTLV proteins p24 **A**, p19 **B**, and p15 **C**. Competition radioimmunoassays were performed with ^{125}I-labeled HTLV p24, p19, or p15, and limiting dilution of the Caribbean normal serum #81-001. Serial dilutions of the labeled antigens starting with 100 ng protein for viral and 50 µg of protein for cellular extracts were preincubated with the serum of 1 h at 37° C. Then 8,000–10,000 cpm of labeled proteins was added and precipitations were performed as under "Methods". ○, HUT102; □, HUT78; ◩, normal human T cells; ●, HTLV; ■, SSV; △, BaEV; M7; ▽, MPMV; ▲, BLV; ▼, FeLV; ◇, R-MuLV

A summary of the results of immunological screening of sera of patients with cutaneous T-cell leukemias/lymphomas (CTCL) in the United States, T-LCL in the Caribbean, and adult T-cell leukemia (ATL) in Japan are given in Table 1. Sera of normal relatives of HTLV-positive patients along with unrelated normal donors were also analyzed for antibodies. HTLV-related T-cell malignancies were found only very rarely in the United States and Europe. Accordingly, antibodies to HTLV proteins were only detected in 2 of 245 sera of CTCL patients that we analyzed. Both the positive cases were also positive for virus isolation. We should note, however, that there may be a significantly higher number of HTLV-positive cases of CTCL than indicated by these numbers because C. Saxinger in our group has found HTLV antigens in sera of some patients included in the antibody-negative CTCL group. In addition, there was a few other United States patients of miscellaneous diagnosis that were positive for antibody to HTLV ([2]; Gallo et al., to be published). So far only rare (< 0.5%) random normal donor in the United States has been found to be antibody positive to HTLV. In contrast, 100% of all the T-LCL sera from the Caribbean and 85% of the ATL sera from Japan have antibodies to HTLV. In Japan, ATL appears in geographical clusters. In these endemic areas about 10% of the normal population carry serum antibodies while only 2% were positive in nonendemic areas of Japan. In the Caribbean islands, the proportion of the random healthy donors positive was about 4%. Among the healthy

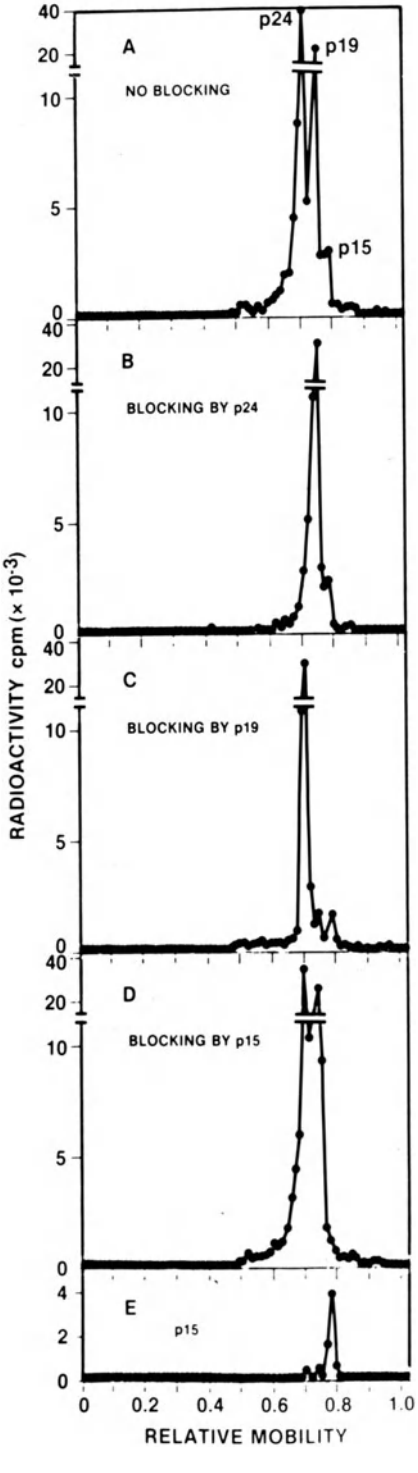

Fig. 3A–E. SDS-polyacrylamide gel electrophoretic analysis of purified and labeled HTLV proteins precipitated by serum #81-001. 100,000 cpm each of p24 p19, and p15 were mixed and incubated with 10 µl serum #81-001, and precipitations were made as described in Fig. 1. The precipitate was washed twice in 5 ml Buffer C, and analyzed by 12% SDS-polyacrylamide gel electrophoresis **A**. To determine the specificity of individual human antibodies for p24, p19, or p15, the serum was also preabsorbed for 3 h at 37° C with 100 ng purified unlabeled p24 **B**, 500 ng p19 **C**, or 500 ng p15 **D** before the mixture of the labeled proteins was added, and precipitations were performed and analyzed as above. A separate precipitate of labeled p15 by 20 µl serum #81-001 was analyzed in **E**, to give additional proof for the existence of the p15 peak in **A–D**. These data show that the purified protein preparations are free of major contaminants and that p24, p19, and p15 do not crossreact

Serum donors	Antibodies to HTLV	
	No. positive/ No. tested	% positive
United States CTCL patients	2/245[a]	< 1
Healthy relatives of CTCL patients	1/8	13
Random healthy donors, United States	0/181	0
Caribbean Sézary and T-LCL patients	8/8	100
Healthy relatives of Caribbean patients	3/16	19
Random healthy donors, Caribbean	12/337	4
Japanese ATL patients	29/34	85
Healthy relatives of ATL patients	13/31	42
Random healthy donors, nonendemic areas	9/509	2
Random healthy donors, endemic areas	39/404	10

[a] Recently, C. Saxinger et al. (unpublished) found HTLV antigen in sera of some of the CTCL antibody-negative patients

Table 1. Prevalence of natural antibodies to HTLV in sera of patients with malignancies of mature T cells, their healthy relatives, and random normal donors

donors, the group that has the highest incidence of serum antibodies to HTLV was the relatives of leukemic patients who are known to be virus positive. Thus out of eight relatives of the two CTCL patients in the United States that were screened, one was positive. Similarly, 42% positivity was found among relatives of Japanese ATL patients and 19% among relatives of Caribbean T-LCL patients. These values are significantly higher than the normal incidence in the population in the respective geographical locations. Among these relatives who were seropositive were spouses, parents, children of either sex, brothers, and sisters, indicating a mode of horizontal transmission of the virus. While those living in the endemic areas could have been exposed to the virus outside the family, the significantly elevated incidence among relatives of virus-positive leukemic patients is a clear reflection of the increased exposure to the virus within the family over the background exposure in the respective neighborhood.

There have been a few cases of non-T-cell malignancies in which antibodies to HTLV have been detected [2]. At least some of them are known to be from ATL-endemic areas, while precise information is lacking on some other caces. Fortuitous infection with HTLV cannot be ruled out in these cases. The available evidence points to a correlation between HTLV infection and a group of adult T-cell malignancies.

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General Summary

Haematology and Blood Transfusion Vol. 28
Modern Trends in Human Leukemia V
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General Summary of the Meeting

J. P. Levy

I will not try to summarize the whole meeting, which has covered the whole scope of leukemia research. The progress in therapy has been overviewed by E. Henderson, so that I will limit my summary to the following questions:

1. Have there been any new developments in leukemia etiology?
2. What has been the progress in elucidating the mechanism of malignant transformation of hematopoietic cells?
3. How well do we understand the nature of leukemic cells?
4. What is the role of tumor immunology in leukemia research?

A. Have There Been Any New Developments in Leukemia Etiology?

It now seems clear that multiple factors are involved in the etiology of leukemias and cancers, including X-rays, chemical carcinogens, and viruses, and even that leukemias can also occur by "spontaneous" mutation without the participation of any of these agents. We are no longer looking for *the* human leukemia virus. Curiously enough, this is just the moment when, after repeated misjudgments over 20 years, a C-type virus of probable human origin has been described. What we know about this human T leukemia virus (HTLV) is still limited, but it appears from the presentations of B. Gallo's group that:

1. It must be a C-type retrovirus.
2. It is different from any previously described agent.
3. It is possibly a human virus. Obviously further studies are necessary to establish this point definitely, but the present information supports this conclusion.
4. It human, it is an exogenous virus, not present in the human genome.
5. Several isolates have been characterized in different areas of Asia and America.
6. There are good arguments that it might be a leukemia virus; notably the epidemiology showing a relationship between T lymphomas and the presence of specific antibodies in patients and related people, the sticking association with a pathology of T cells only and, as reported here, the possible in vitro transforming activity of HTLV for human cord blood T cells.

This suggests that two different human malignant hematopoietic disease could be associated with viruses: Pre-B cell malignancies of the Burkitt type with EBV and certain T lymphomas or leukemias with HTLV.

It this is confirmed, several questions will remain to be solved.

First: are these viruses transforming or could they be only promoting factors as possible for EBV. Second: why are these malignancies so infrequent, since they represent only a small percentage of human leukemias? Is it really due, as probable, to the existence of a strong immune response directed against the viral antigens? If yes, the reason why the immune response could be deficient in the clusters of HTLV-associated diseases will remain to be determined as well as the possible role of cocarcinogens. These questions have long been posed with regard to EBV. Another point will be the possibility of vaccinating, which could be difficult for technical as

well as economic reasons. Moreover, how do we decide who should be vaccinated against such an unfrequent disease? Epidemiological studies with the aim of defining high-risk patients and possible co-carcinogens therefore appear very important for the future prevention of these virus-associated malignancies.

B. What Has Been the Progress in Elucidating The Mechanism of the Malignant Transformation of Hematopoietic Cells?

Three groups of information have been presented in this meeting concerning transformation by virus-associated *onc* genes (*v-onc*), by cellular *onc* genes (*c-onc*), and without *onc* gene.

I. Transformation by *v-onc*

That *v-onc* is responsible for the malignant transformation induced by oncogenic viruses is clear, as confirmed in this meeting by the results of Bister et al., for example; but the mechanism of the activity of the 15–20 *v-onc* presently known remains to be established. Some of them produce a protein with tyrosine-phosphorylase activity. Their target protein seems to be related to the cell membrane or cytoskeleton, but we are still ignorant of its precise nature. It has not even been definitely demonstrated that tyrosine phosphorylation is related to carcinogenesis. One may suppose that this kind of *onc* gene product either inhibits the action of a regulator exogenous factor or that it mimics its effects inside the cell. On the other hand, K. Moelling and her colleagues have shown that the *myc* product is a DNA-binding protein, and they reported that the *Erb*^{a} gene product could have a third mechanism of action which involves neither a protein kinase nor a DNA-binding protein. This shows that the malignant transformation might occur as the result of different molecular lesions due to various kinds of *onc* gene products.

It also appears that a common mechanism might exist for DNA- and RNA-virus-induced transformations as suggested by the observation that the *myc* product and SV40T antigen are both DNA-binding proteins, while it has been suggested that a polyoma virus antigen could be a protein kinase like most of the *onc* gene products of RNA viruses. W.S. Rigby has shown that normal cellular proteins are induced by SV40. One may suppose that among these proteins, some are especially important for transformation, and one may imagine that some of them can be involved whatever the inducing virus if a chain of molecular events is altered at different steps by various carcinogens.

II. Transformation by *c-onc*

Several papers have recently suggested that leukemia viruses not possessing an *onc* gene might be leukemogenic by derepressing a *c-onc*. We know that *c-onc* and *v-onc* are very similar and could be identical, as illustrated here by the presentations of F. Wong-Staal et al., Vande Woode et al., and Dalla Favera et al. It has been shown also that *c-onc* can be expressed in experimental as well as human tumors. What does this mean?

In animal species from which *v-onc* and their *c-onc* counterpart have been initially described, the question at first appeared simple, following the observation that the derepression of *c-myc* by an upstream integrated viral LTR able to induce the transformation. As discussed in this meeting, notably by F. Vande Woode and by P. Duesberg, the phenomenon seems less clear now that the viral LTR can be integrated not only upstream, but also downstream to the *c-onc,* reading seems to occur in both directions, and the LTR can be integrated relatively far from the *c-onc*. What is the significance of *c-myc* expression in these condition? Is it really related to cancer? How many genes with possible *onc* characters can be expressed which are not detected because we do not possess their *v-onc* counterpart? The most important question has in fact been discussed by P. Duesberg, i.e., are *c-onc* and *v-onc* truly equivalent? It is generally supposed that they are identical and that quantitative differences in the expression of *onc* genes products are sufficient to explain malignancies. It cannot definitely be excluded, however, that qualitative differences still exist between *v-onc*

and *c-onc*. Minor differences in their sequences, as illustrated by Papas et al., might be responsible for the oncogenic properties of *v-onc*. In addition, the role of the so-called "introns" which exist in *c-onc* and not in *v-onc* might be important for a cellular function of *c-onc* that we are still ignorant of, and it would be very important to know what the normal role of the *c-onc* is in differentiation or for any other function. Are they capable of something which *v-onc* is not? Finally, many *v-onc* produce a protein which is not really equivalent to the *c-onc* product since it is associated with viral sequencies coming from the *gag* gene for example, and we do not know whether this association could modify the function or not. On the whole *c-onc* genes are possibly responsible for cancer due to their quantitatively abnormal expression. Many arguments support this idea, but the possibility still remains that *v-onc* could be the abnormal equivalent of *c-onc*, expressing an oncogenic potency which does not exist for *c-onc*. The observation that *c-mos* associated with a viral LTR becomes oncogenic strongly supports the quantitative hypothesis as shown by Vande Woode, but why *c-src* or *Hv-mos* do not function in the same conditions still needs explaining. It is probable that the problem will not be solved until we know the normal function of *c-onc* genes, which seems to be so conservative that they exist, at least for one of them *c-src* from sponges to human beings, as illustrated by F. Anders. The solution of this problem must be of importance for future developments in cancer therapy.

Another approach of the role of *c-onc* has been reported in this meeting by F. Wong-Staal et al., Della Favera et al., Rübsamen et al., and Vande Woode et al., who have studied the expression of known *c-onc* in human tumors. It seems that *myc, abl,* and *Hv-mos* (the *c-onc* corresponding to the *v-onc* of Harvey virus) can be expressed in any kind of tumor. On the other hand, *myb* was found in poorly differentiated tumors only, *src* was rarely expressed but present for example in some breast cancers, and the expression of *sis* appeared exceptional. It is difficult to make conclusions about the significance of these phenomena, expression being either occasional without clear tissue specificity, or regular in all kinds of tumor. Moreover, normal tissues are able to express the same genes at a relatively high level.

Other groups are looking for *c-onc* genes by transfection of human tumor DNA in NIH 3T3 cells. M.A. Lane and her colleagues have shown that some highly conservative genes might exist in human as well as in murine tumors, with conservation of restriction sites which could be specific for B- or T-cell malignancies, and even more precisely for poorly differentiated, intermediate, or mature cells of each lineage. These genes are different from the known *c-onc* genes which have been tested. On the other hand, Dautry et al. reported the expression of the Harvey gene in bladder carcinoma, that of the Kirsten gene in colonic cancer, and that of another gene in HL 60 leukemic cells and possibly also in Burkitt tumors. HL 60 cells have been shown also to express *c-myc* (Della Favera et al.), which, however, appeared not to be expressed in other acute promyelocytic leukemias. These results are fascinating since they suggest the possible role of at least some of these genes in human malignancies, but their interpretation remains difficult. It has previously been shown by Cooper et al. that the human normal DNA contains genes which are able to transform 3T3 cells. On the contrary, the genes described by M.A. Lane are apparently not found in normal DNA, which could suggest that they are not the exact equivalent of the *c-onc*. On the other hand, such experiments are presently limited by technical problems, and further studies using other target cells from other tissues and other animal species, including man, are necessary for progress. Another question is related to the possible selection in such experiments of *c-onc* genes of which the corresponding *v-onc* have been isolated precisely by their ability to transform murine 3T3 cells. Does their isolation in these conditions really suggest that they play a role in the original human tumor? A larger number of experiments demonstrating tumor specificity of these genes, as suggested by M.A. Lane, would be at least necessary. At the present time, these observations are remarkable, but no conclusion can be drawn. By the way, it can be observed that the observation by Dautry et al. that Harvey and Kir-

sten gene equivalents transform NIH 3T3 cells would support the previously discussed idea that *c-onc* are transforming and qualitatively equivalent to *v-onc*.

III. Transformation Without *onc* Genes

B. Haseltine and P. Fischinger have presented results obtained with murine leukemia virus which suggest possible oncogenic transformation without *onc* genes – more precisely, without a direct intervention of *onc* genes. Weissman has previously suggested that the permanent stimulation of T cells by a C-type virus which is their specific antigen might favor the appearance of leukemia-specific chromosomal abnormalities. Experimental data supporting this idea have been obtained in the group of J. Ihle. The observation by P. Fischinger that there are a very large number of different MCF-tpe *gp* recombinants of the Moloney virus supports the idea that multiple T-cell clones of different specificities might be involved in this phenomenon, perhaps explaining the diversity of leukemia which is produced. On the other hand, the study of AKR leukemia viruses by B. Haseltine and his group shows that the oncogenic potency of one of these agents is related to a very precise mutation near the 3′ end. This suggests something wrong on the intracellular portion of p15E. How can it explain malignancy? Could the proteins of the viral envelope be related to normal cell surface proteins? It has been shown, for example, that p15E of the Moloney virus would be the receptor for $C1_q$, and it is possible that cellular proteins of the gp70 family might be involved in cellular interactions, notably in the thymus. Does an abnormal protein induce abnormal cell interaction with chronic stimulation and eventually the possible induction of *c-onc* or any other genetic abnormality?

In conclusion, it is still impossible to draw conclusions about the mechanism of viral oncogenesis, and even more difficult to propose to general theory of carcinogensis, but the progress has been remarkable in the last 3 years, and such a theory appears at least possible in the next few years.

One must say that in addition to the data obtained by virologists and molecular biologists, very important information has been obtained in the last 3- to 4-year period by cytogeneticists. This point has not been developed in this meeting, but the remarkable advances in chromosome isolations presented by Dr. Young, with the possibility of separating the normal and the translocated chromosomes of one pair, will provide an extremely useful clue in correlating the morphological and biochemical lesions of chromosomes and in determining, in cases where there is a leukemia-specific translocation, which genetic sequences are involved.

Altogether, these advances suggest for the first time that an understanding of what a cancer cell is at the biochemical level will be soon possible.

C. How Well Do We Understand the Nature of Leukemic Cells?

The first point which is now definitely clear is that any leukemic cell has a normal counterpart. This has already been strongly suggested by the recent progress in cytology and pathology, and this is now clearly demonstrated by the use of different markers, including notably monoclonal antibodies as shown by several presentations at this meeting. A remarkable clarification of the classification of the malignant diseases of hematopoietic origin has been recently achieved, as clearly shown here by M. Greaves and also by D. Cooper. Up to recently, however, two cases have remained mysterious: hairy cell leukemia and Hodgkin's disease. As far as hairy cell leukemia is concerned, it appears possible that the normal counterparts of leukemic cells belong to a new minor cellular population of unknown function. Similarly, we have learned here from Dr. Stern and Dr. Diehl that the Reed-Sternberg cell of Hodgkin patients would not belong to any of the previously described lineages. It would be the malignant counterpart of a normal cell present in the external region of lymphoid follicules, as well as in spleen and bone marrow. Since there are now permanent cell lines which are apparently de-

rived from Sternberg cells and specific monoclonal antibodies, it will probably be possible to study the exact nature and function of this new cell, which apparently is not a macrophage but possesses several properties generally supposed to be associated with macrophages, including the production of IL1 and CSF, the expression of Ia antigens, and an accessory cell function in immunological responses. The results reported here are very important for the understanding of Hodgkin's disease, which is the last frequent malignant hemopathy of which the origin remained unclear with so contradictory conclusions from different groups.

It appears not only that leukemic cell lines have a normal counterpart, but also that heir phenotype can be normal, as far as the presently known markers are studied. As pointed by M. Greaves, it is probable that normal progenitors possess all the genetic information necessary for the expression of leukemic properties. The leukemic cells seem remarkable, mainly by an abnormal stabilization of their phenotype at a given stage, with uncoupling of growth and differentiation. The appearence of some phenotypic abnormalities in the leukemic cell is frequent, but it might be a late event. Furthermore, the reversion of leukemic cells to normal cells is possible, and the results reported by Dr. Metcalf suggest a possible reprogrammation of leukemic cells with normal differentiation under the influence of biological soluble factors. This has also been illustrated by M. Moore using the soluble HDIF, and the possible effect of chemical substances like retinoids and dihydroxychole calciferol. From all these observations, it appears that an apparently normal functional adult cell can derive from a leukemic cell. Is this compatible with the results obtained by molecular biologists? The answer is probably yes, since the genetic lesion of malignant cells, whether related to the expression of *c-onc* genes or not, could be finally responsible for an abnormal reaction to soluble factors with uncoupling of growth and differentiation. A continuous treatment by soluble factors would therefore be necessary to maintain the normal differentiation of leukemic cells, which would be cured at the phenotypic but not genotypic level, unless a real reprogrammation of the cells could be induced by soluble factors as suggested here by Dr. Metcalf.

It must be pointed out that we are still almost completely ignorant of the exact reason why a normal cell becomes a leukemic cell. It could be hypersensitive to growth factors, which could also be produced in excess in the surrounding of progenitor cells by the abnormal progenitors themselves or by other cells. One can also imagine that leukemic cells are less sensitive to differentiation factors. The only point which is clear is that this cell is not a monster.

What soluble factors are involved in these phenomena? This is still impossible to answer since we do not know exactly the number and the role of soluble factors in normal granulopoiesis for example. From the presentations of Dr. Metcalf and Dr. Moore, it appears that there is a family of CSF probably acting at several levels, with variable degrees of specificity, but the exact number of these factors is still unclear. Moreover, there is a very important point: are the same or different factors involved in cell growth and cell differentiation? It would be perhaps easier to understand leukemia if different factors were involved, but purification and molecular cloning of the different CSF and related factors will probably be necessary to answer this question. They will also be necessary before hypothetical use of these factors for leukemia treatment. The results presented at this meeting have shown that there is reasonable hope that this hypothesis will be confirmed in the future.

D. What is The Role of Tumor Immunology in Leukemia Research?

At this meeting we have had some excellent presentations in basic immunology. I cannot summarize these papers, which in fact were not directly related to leukemia. One must say, however, that major progress in understanding leukemia and its treatment will probably occur as a consequence of a better knowledge of cell membrane antigens, and the results which have been reported and discussed by H. Ploegh and by

C. Terhorst on the biochemistry of histocompatibility and differentiation antigens, or the progress in the understanding of these antigens at a genetical level, as presented by E. Weiss and by N. Mitchison, are opening up new areas in this research.

The part on specific tumor immunology was not very large at this meeting, and this is not surprising since some disappointments have followed the enthusiastic period that tumor immunology went through some years ago. The research on tumor-specific antigens in human beings has not been very fruitful, and this is in agreement with the observations about the nature of leukemic cells as extensively discussed during these 3 days. It is probably not surprising that no specific antigen exists on tumor cells if these cells have a phenotype similar to that of normal cells, and if they result only from an uncoupling between growth and differentiation. If *c-onc* genes are involved, one can imagine that their products would be nonantigenic for the host. Nevertheless, a virus-specific immune response must exist when a virus is present, and the HTLV-associated leukemias will probably lead to new interest in tumor immunology.

A marginal observation concerning these leukemias has been reported by B. Gallo which deserves further discussion. It seems that they can express foreign class I HLA activity, recalling previously reported observations in murine systems. The remarkable results reported here by E. Weiss on the cloning of *HLA genes* do not support the hypothesis that normally silent histocompatibility genes are depressed in leukemic cells as sometimes suggested. One may imagine minor posttranslational modifications of HLA molecules, or that the association of these molecules with viral products would mimic allospecificities. Whatever its nature, this phenomenon could be useful for leukemia rejection, and it would be interesting to know whether it is specific for virus-associated systems. This was not clear in the murine system due to the high level of contamination by C-type viruses of any murine tumor.

Much attention has been paid in recent years to nonspecific tumor immunology and especially to natural killer cells. Initially known only by their apparently nonspecific activity on tumor cells, they have been progressively better defined morphologically and by their markers in man. Their exact nature however, remains, unclear, and they have recently been described as T-cell precursors, or monocytes, or as a special lineage, and the existence of several kinds of NK cells with different markers has been described. An overview of NK cells has been given here by H. Wigzell, and it appears that besides well-defined NK cells other cells may acquire and NK activity. Cytolytic T cells (CTL), for example, obtained by cloning procedures can be NK cells, but the point is that there are two different structures of these T cells reacting with the target antigen of CTL and the target molecule of the NK activity, respectively. We are still ignorant of this structure that NK cells are able to recognize. From H. Wigzell's data, the situation is less simple than generally supposed: poorly differentiated cells in general are good targets, but the differentiation of these cells can either decrease or increase the sensitivity. Some correlation exists between an increase in the content of sialic acid and glycolipids and a decrease in NK sensitivity. The resistance to NK cells is, however, always relative, and apparently resistant tumor cells can be lysed with stronger NK cells. The main problem remains: we do not know whether NK cells are really protective in vivo against tumors: this is suggested in some cases but not definitely demonstrated. Also we are still ignorant of whether NK cells can have a normal regulatory function, but it appears that they kill CFUs, which can support this fascinating hypothesis.

Finally, a kind of revenge of tumor immunology has been well illustrated during the last day of this meeting. Monoclonal antibodies specific for differentiation antigens expressed normally on leukemic cells represent a new possibility in leukemia therapy, either as vectors of drugs or toxin as shown here for example by P. Thorpe, or to eliminate residual leukemic cells before a bone marrow autograft, as illustrated remarkably by the Sydney Farber Group. On the other hand, bone marrow allografts now represent one of the major components of leukemia treatment, and from the results of Dr. Thomas it is clear that more and more patients will be grafted in

future years. Here again, the progress of basic immunology will become a determining factor since the problem will be to improve the treatment of the graft-versus-host reaction (GVHR), which is the primary cause of death in AML. Nevertheless, the results observed with ALL suggest that GVHR is probably useful in eliminating leukemic cells; we will perhaps have to learn what the benefit of GVH is.

The general conclusion of this meeting is therefore very optimistic. Cancer research has recently seen a relatively black period, but a new period is now beginning. We have at the same time very good progress in the understanding of the leukemic cell at molecular as well as cellular levels, and really new approaches in therapy.

The situation has never been so stimulating for scientists.

Subject Index

Haematology and Blood Transfusion

Supplement volumes to the journal „Blut"

Editors: H. Heimpel, D. Huhn, G. Ruhenstroth-Bauer, W. Stich

A selection

Modern Trends in Human Leukemia IV

Latest Results in Clinical and Biological Research Including Pediatric Oncology
Wilsede Joint Meeting on Pediatric Oncology I, Hamburg, June 20–21, 1980

Editors: R. Neth, R. C. Gallo, T. Graf, K. Mannweiler, K. Winkler

1981. 252 figures, 118 tables. XXV, 557 pages (Volume 26)
ISBN 3-540-10622-7

This book contains a report on the Wilsede meeting held in Germany in June, 1980. The meeting included the Frederick Stohlman lectures on leukemia, held by G. Klein on the relative role of viral transformation and cytogenetic changes in human and mouse lymphomas, and by H. Kaplan on the biology of Hodgkin's disease. The contributions are devided into clinical cytogenetic, cell biologic, immunologic, virologic, and molecularbiologic sections. Also included is the Wildese Joint Meeting on Pediatric Oncology I.
The book will be of value to both clinicians and researchers in many fields of oncology, as well as for investigators and students interested in human leukemia.

Modern Trends in Human Leukemia III

Newest Results in Clinical and Biological Research

9th Scientific Meeting of „Gesellschaft Deutscher Naturforscher und Ärzte"

Together with the „Deutsche Gesellschaft für Hämatologie",
Wilsede, June 19–23, 1978

Editors: R. Neth, R. C. Gallo, P.-H. Hofschneider, K. Mannweiler

1979. 171 figures, 128 tables. XXII, 599 pages (Volume 23)
ISBN 3-540-08999-3

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Modern Trends in Human Leukemia II

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International Symposium on Aplastic Anemia
July 19 - 22,1978, Schloß Reisensburg

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International Symposium of the Institut für Hämatologie, GSF, October 28 - 30, 1976, Neuherberg/München

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