Haematology and Blood Transfusion
25
Hämatologie und Bluttransfusion

Edited by
H. Heimpel, Ulm · D. Huhn, München
G. Ruhenstroth-Bauer, München

Immunobiology of Bone Marrow Transplantation

International Seminar
of the Institut für Hämatologie, GSF, Munich
under the auspices of the European Communities
March 8–10, 1979, Neuherberg/München

Edited by
S. Thierfelder, H. Rodt and H. J. Kolb

With 123 Figures and 123 Tables

Springer-Verlag
Berlin Heidelberg New York 1980

Professor Dr. Stefan Thierfelder
Dr. Hans Rodt
Dr. Hans Jochem Kolb
Institut für Hämatologie der Gesellschaft
für Strahlen- und Umweltforschung
Landwehrstraße 61
D-8000 München 2, Bundesrepublik Deutschland

Supplement to

BLUT – Journal for Blood Research

Organ of the "Deutsche Gesellschaft für Hämatologie und Onkologie der Deutschen Gesellschaft für Bluttransfusion und Immunohämatologie" and of the "Österreichischen Gesellschaft für Hämatologie und Onkologie"

Edited by H. Heimpel, Ulm. D. Huhn, München. G. Ruhenstroth-Bauer, München

ISBN-13: 978-3-540-09405-0 e-ISBN-13: 978-3-642-67319-1
DOI: 10.1007/978-3-642-67319-1

Typesetting, printing and binding: Kösel GmbH & Co., Kempten
2329/3321/543210

Contents

6 Autologous Bone Marrow Transplantation

7 Conditioning of Bone Marrow Recipients

8 Allogeneic Bone Marrow Transplantation for the Treatment of Leukemia, Aplastic Anemia and Immunodeficiency

9 Summing up

Contributors to the Meeting

Arnold, Renate, Dept. für Innere Medizin der Univ., Abt. Hämatologie, Steinhövelstr. 9, 7900 Ulm, West-Germany

Atkinson, K., Univ. of Washington, School of Medicine, Seattle, WA. 98195, USA

Bacigalupo, A., Ospedali di Civili Genova, Div. di Ematologia e di Immunologia Clinica, 16132 Genova, Italy

Bekkum, D. W. van, Radiobiological Inst. TNO, 151 Lange Kleiweg, 2280 HV Rijswijk, Netherlands

Bernard, J., U.E.R. d'Hématologie-Paris VII, Centre Hayem, 2, Place du Dr. Fournier, 75010 Paris, France

Beschorner, W. E., John Hopkins Univ. School of Medicine, Baltimore, MD. 21205, USA

Blasecki, J. W., Medical College of Wisconsin, 948, North 12th Street, Milwaukee, WI. 53233, USA

Bodenberger, U., Abt. Immunologie, Institut für Hämatologie, Gesellschaft für Strahlen- und Umweltforschung, Landwehrstr. 61, 8000 München 2, West-Germany

Boecker, W. R., Westdeutsches Tumorzentrum, Univ.-Klinik der GHS Essen, Hufelandstr. 55, 4300 Essen, West-Germany

Boiron, M., U.E.R. d'Hématologie-Paris VII, Centre Hayem, 2, Place du Dr. Fournier, 75010 Paris, France

Bortin, M. M., Medical College of Wisconsin, 948, North 12th Street, Milwaukee, WI. 53233, USA

Braine, H. G., Oncology Center, The John Hopkins Univ. School of Medicine, Baltimore, MD. 21205, USA

Buriot, D., Dept. de Pédiatrie, Hôpital des Enfants Malades, 149, Rue de Sèvres, 75730 Paris, France

Coccia, P., Dept. of Pediatrics, Univ. of Minnesota, Minneapolis, MN. 55455, USA

Colombani, J., Laboratoire d'Immuno-hematology, INSERM W 39, Hôpital St. Louis, Paris, France

Deeg, H. J., Univ. of Washington School of Medicine, Seattle, WA. 98195, USA

Devergie, A., U.E.R. d'Hématologie-Paris VII, Centre Hayem, 2, Place du Dr. Fournier, 75010 Paris, France

Dexter, T. M., Christie Hospital and Holt Radium Inst., Paterson-Laboratories, Manchester, M20 9BX, Great Britain

Dicke, K. A., Anderson Hospital and Tumor Inst., 6723 Bertner, Houston, TX. 77030, USA

Dickmeiss, E., State Univ. Hospital, Copenhagen, Denmark

Duhamel, G., Service des Maladies du Sang, Hôpital St. Antoine, Paris, France

Dupont, B., Sloan-Kettering Inst. for Cancer Research, 1250 First Av., New York, NY. 10021, USA

Durandy, A., Dept. de Pédiatrie, Hôpital des Enfants Malades, 149, Rue de Sèvres, 75730 Paris, France

Dutreix, A., Inst. Gustave Roussy, Dept. de Radio-physique, 16 bis, Av. Paul Vaillant Couturier, 94800 Villejuif, France

Dutreix, J., Inst. Gustave Roussy, Dept. de Radio-physique, 16 bis, Av. Paul Vaillant Couturier, 94800 Villejuif, France

Economou, J. G., Oncology Center, The John Hopkins Univ. School of Medicine, Baltimore, MD. 21205, USA
Elfenbein, G. J., Oncology Center, The John Hopkins Univ. School of Medicine, Baltimore, MD. 21205, USA
Farthing, M. G., Dept of Gastroenterology, St. Bartholomew's Hospital, West Smithfield, London E. C. 1, Great Britain
Flad, H. D., Zentrum für Klinische Grundlagenforschung der Univ., Oberer Eselsberg, 7900 Ulm, West-Germany
Fliedner, T. M., Abt. für Klinische Physiologie der Univ., Oberer Eselsberg, 7900 Ulm, West-Germany
Friedenstein, A. J., Gamaleya Inst. of Epidemiology and Microbiology, USSR Academy of Medical Sciences, Moscow, D-98, USSR
Gluckman, E., U.E.R. d'Hématologie-Paris VII, Centre Hayem, 2, Place du Dr. Fournier, 75010 Paris, France
Goldmann, S. F., Tissue Typing Labor, Abt. Klinische Physiologie, Steinhövelstr. 9, 7900 Ulm, West-Germany
Good, R. A., Sloan-Kettering Inst. for Cancer Research, 1250 First Av., New York, NY. 10021, USA
Gorin, N. C., Centre Hospitalier et Universitaire St. Antoine, 184, Rue du Fauburg St. Antoine, 75571 Paris, France
Graham, T. C., Univ. of Washington School of Medicine, Seattle, WA. 98195, USA
Griscelli, C., Dept. de Pédiatrie, Hôpital des Enfants Malades, 149, Rue de Sèvres, 75730 Paris, France
Grosse-Wilde, H., Abt. Immunologie, Institut für Hämatologie, Gesellschaft für Strahlen- und Umweltforschung, Landwehrstr. 61, 8000 München 2, West-Germany
Haas, R. J., Univ.-Klinik im Dr. von Haunerschen Kinderspital, 8000 München 2, West-Germany
Halle-Pannenko, Olga, Inst. de Cancerologie, Groupe Hospitalier Paul Brousse, 14, Av. Paul Vaillant Couturier, 94800 Villejuif, France
Hansen, Grete S., State Univ. Hospital, 2100 Copenhagen, Denmark
Heimpel, H., Dept. für Innere Medizin der Univ., Steinhövelstr. 9, 7900 Ulm, West-Germany
Hellriegel, K.-P., 1. Med. Univ.-Klinik, Joseph-Stelzmannstr. 9, 5000 Köln 41, West-Germany
Hess, A. D., John Hopkins Univ. School of Medicine, Baltimore, MD. 21205, USA
Hoffmann-Fezer, G., Abt. Immunologie, Inst. für Hämatologie der Gesellschaft für Strahlen- und Umweltforschung, Landwehrstr. 61, 8000 München 2, West-Germany
Hunt, S. V., Cellular Immunology Unit. Sir William Dunn School of Pathology, Oxford Univ., South Parks Road, Oxford 0X1 3RE, Great Britain
Ivanyi, J., Dept. of Experimental Immunobiology, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, Great Britain
Janka, G., Inst. für Hämatologie, Gesellschaft für Strahlen- und Umweltschutz, Landwehrstr. 61, 8000 München 2, West-Germany
Johnson, R. J., Oncology Center, The John Hopkins Univ. School of Medicine, Baltimore, MD. 21205, USA
Kaizer, H., Oncology Center 3–121, The John Hopkins Hospital, 601, North Broadway, Baltimore, MD. 21205, USA
Kay, H. E. M., Dept. of Clinical Pathology, The London Marsden Hospital London & Surrey, Fulham Road, London SW 6JJ, Great Britain
Kersey, J. H., Dept. of Pediatrics, Univ. of Minnesota, Minneapolis, MN. 55455, USA
Kim, T., Dept. of Pediatrics, Univ. of Minnesota, Minneapolis, MN. 55455, USA

Kindred, Berenice, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 6900 Heidelberg 1, West-Germany
Kleihauer, E., Abt. Hämatologie, Kinderklinik der Univ., Prittwitzstr. 43, 7900 Ulm, West-Germany
Kolb, H. J., Abt. Immunologie, Institut für Hämatologie, Gesellschaft für Strahlen- und Umweltforschung, Ingolstädter Landstr. 1, 8042 Neuherberg, West-Germany
Koning, J. de, Dept. of Pediatrics. Univ. Hospital, Rijnsburgerweg 10, 2333 AA Leyden, Netherlands
Krivit, W., Dept. of Pediatrics, Univ. of Minnesota, Minneapolis, MN. 55455, USA
Leventhal, Brigid G., Oncology Center, The John Hopkins Univ. School of Medicine, Baltimore, MD. 21205, USA
Levitt, S., Dept. of Pediatrics, Univ. of Minnesota, Minneapolis, MN. 55455, USA
Lint, M. T. van, Dept. of Hematology, Ospedale San Martino, 16132 Genova, Italy
Marmont, A. M., Dept. of Hematology, Ospedale San Martino, 16132 Genova, Italy
Mathé, G., Inst. de Cancérologie, Groupe Hospitalier Paul Brousse, 14, Av. Paul Vaillant Couturier, 94800 Villejuif, France
Maurer, P. H., Dept. of Biochemistry, Thomas Jefferson Univ., Philadelphia 19107, USA
McCredie, K. B., Anderson Hospital and Tumor Inst., Dept. of Developmental Therapeutics, Houston, TX. 77030, USA
Mingari, M. C., Inst. of Microbiology, Univ. of Genova, 16132, Genova, Italy
Moretta, L., Inst. of Microbiology, Univ. of Genova, 16132, Genova, Italy
Müller-Hermelink, H. K., Hygiene-Inst. der Univ., Brunswikerstr. 2–6, 2300 Kiel, West-Germany
Müller-Ruchholtz, W., Abt. Immunologie, Hygiene-Inst. der Univ., Brunswikerstr. 2–6, 2300 Kiel, West-Germany
Muller, J. Y., Centre National de Transfusion Sanguine, Paris, France
Munoz, L. L., Oncology Center, The John Hopkins Univ. School of Medicine, Baltimore, MD. 21205, USA
Munro, A., Dept. of Pathology, Univ. of Cambridge, Tennis Court Road, Cambridge, Great Britain
Nesbit, M. E., Dept. of Pediatrics, Univ. of Minnesota, Minneapolis, MN. 55455, USA
Netzel, B., Univ.-Klinik im Dr. von Haunerschen Kinderspital, 8000 München 2, West-Germany
Niethammer, D., Abt. für pädiatrische Hämatologie, Univ.-Kinderklinik, Rümelinstr. 23, 7400 Tübingen 1, West-Germany
Nowrousian, M. R., Westdeutsches Tumorzentrum, Univ.-Klinik der GHS Essen, Hufelandstr. 55, 4300 Essen, West-Germany
Öhl, S., Westdeutsches Tumorzentrum, Univ.-Klinik der GHS Essen, Hufelandstr. 55, 4300 Essen, West-Germany
O'Leary, Maura, Dept. of Pediatrics., Univ. of Minnesota, Minneapolis, MN. 55455, USA
O'Reilly, R. J., Sloan-Kettering Inst. for Cancer Research, 1250 First Av., New York, NY. 10021, USA
Peters, L., Anderson Hospital and Tumor Inst., 6723 Bertner, Houston, TX. 77030, USA
Pflieger, H., Dept. für Innere Medizin der Univ. Abt. Hämatologie, Steinhövelstr. 9, 7900 Ulm, West-Germany
Piaggio, G., Dept. of Hematology, Ospedale San Martino, 16132, Genova, Italy
Podesta, M., Dept. of Hematology, Ospedale San Martino, 16132, Genova, Italy
Pollack, Marilyn S., Sloan-Kettering Inst. for Cancer Research, 1250 First Av., New York, NY. 10021, USA

Powles, R. L., Leukemia Unit, Royal Marsden Hospital, London & Surrey, London SW 6 JJ, Great Britain
Pritchard, Linda L., Inst. de Cancerologie, Groupe Hospitalier Paul Brousse, 14, Av. Paul Vaillant Couturier, 94800 Villejuif, France
Raffo, M. C., Dept. of Hematology, Ospedale San Martino, 16132, Genova, Italy
Ramsay, Norma K. C., Dept. of Pediatrics, Univ. of Minnesota, Minneapolis, MN. 55455, USA
Rieder, I., Abt. Klinische Hämatologie, Inst. für Hämatologie der Gesellschaft für Strahlen- und Umweltforschung, Landwehrstr. 61, 8000 München 2, West-Germany
Rimm, A. A., Medical College of Wisconsin, 948, North 12th Street, Milwaukee, WI. 53233, USA
Rodt, H., Inst. für Hämatologie der Gesellschaft für Strahlen- und Umweltforschung, Landwehrstr. 61, 8000 München 2, West-Germany
Röllinghoff, M., Inst. für Med. Mikrobiologie, Hochhaus am Augustusplatz, 6500 Mainz, West-Germany
Salmon, C., Centre National de Transfusion Sanguine, Paris, France
Santos, G. W., Oncology Center, The John Hopkins Univ. School of Medicine, Baltimore, MD. 21205, USA
Schaefer, U. W., Westdeutsches Tumorzentrum, Univ.-Klinik der GHS Essen, Hufelandstr. 55, 4300 Essen, West-Germany
Scheulen, M. E., Westdeutsches Tumorzentrum, Univ.-Klinik der GHS Essen, Hufelandstr. 55, 4300 Essen, West-Germany
Schilcher, B., Westdeutsches Tumorzentrum, Univ.-Klinik der GHS Essen, Hufelandstr. 55, 4300 Essen, West-Germany
Schmidt, C. G., Westdeutsches Tumorzentrum, Univ.-Klinik der GHS Essen, Hufelandstr. 55, 4300 Essen, West-Germany
Scholz, S., Kinderpoliklinik der Univ., Pettenkoferstr. 8a, 8000 München 2, West-Germany
Shih, C.-Y., Medical College of Wisconsin, 948 North 12th Street, Milwaukee, WI. 53233, USA
Shin, H. S., Oncology Center, The John Hopkins Univ. School of Medicine, Baltimore, MD. 21205, USA
Slavin, S., Dept. of Medicine A, Hadassah-Hebrew Univ. Hospital, Jerusalem, Israel
Sloane, J. P., Leukemia Unit, Royal Marsden Hospital, London & Surrey, London SW 6JJ, Great Britain
Søeberg, B., State Univ. Hospital, 2100 Copenhagen, Denmark
Spitzer, G., Anderson Hospital and Tumor Inst., 6723 Bertner, Houston, TX 77030, USA
Spooncer, E., Christie Hospital and Holt Radium-Institute, Paterson Laboratories, Manchester M 20 9BX, Great Britain
Stewart, D., Anderson Hospital and Tumor Inst., 6723 Bertner, Houston, TX. 77030, USA
Storb, R., Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA. 98104, USA
Strober, S., Dept. of Medicine and Howard Hughes Medical Inst. Laboratory, Stanford Univ., Stanford, CA., USA
Stursberg, G., Dept. für Innere Medizin der Univ., Steinhövelstr. 9, 7900 Ulm, West-Germany
Sveijgaard, A., Tissue-Typing Laboratory, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark
Thiel, E., Abt. Immunologie, Inst. für Hämatologie der Gesellschaft für Strahlen- und Umweltforschung, Landwehrstr. 61, 8000 München 2, West-Germany
Thierfelder, S., Abt. Immunologie, Inst. für Hämatologie der Gesellschaft für Strahlen- und Umweltforschung, Landwehrstr. 61, 8000 München 2, West-Germany

Thomas, E. D., Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA. 98104, USA
Thomson, S., Anderson Hospital and Tumor Inst. 6723 Bertner, Houston, TX. 77030, USA
Torok-Storb, B., Univ. of Washington School of Medicine, Seattle, WA. 98195, USA
Touraine, J.-L., INSERM U 80 and Clinic of Nephrology, Hôpital E. Herriot, 69374 Lyon, Cedex 2, France
Truitt, R. L., Medical College of Wisconsin, Milwaukee, WI. 53233, USA
Tutschka, P. J., Oncology Center, The John Hopkins Hospital, 601 North Wolfe St., Baltimore, MD. 21205, USA
Veer-Korthof, E. T. van't, Dept. of Pediatrics, Univ. Hospital, Rijnsburgerweg 10, 2333 AA Leyden, Netherlands
Vellekoop, L., Anderson Hospital and Tumor Inst., 6723 Bertner, Houston, TX. 77030, USA
Verma, D. S., Anderson Hospital and Tumor Inst., 6723 Bertner, Houston, TX. 77030, USA
Vetter, U., Dept. für Innere Medizin der Univ., Abt. Hämatologie, Steinhövelstr. 9, 7900 Ulm, West-Germany
Virelizier, J. L., Dept. de Pédiatrie, Hôpital des Enfants Malades, 149, Rue de Sèvres, 75730 Paris, France
Vriesendorp, H. M., Radiobiological Inst. TNO, 151 Lange Kleiweg, 2280 HV Rijswijk, Netherlands
Wagner, H., Inst. für Med. Mikrobiologie, Hochhaus am Augustusplatz, 6500 Mainz, West-Germany
Waldmann, H., MRC Laboratory of Molecular Biology, Univ. Medical School, Hills Road, Cambridge CB2 2QH, Great Britain
Warkentin, Phyllis, Dept. of Pediatrics, Univ. of Minnesota, Minneapolis, MN. 55455, USA
Weel-Sipman, M. H. van, Dept. of Pediatrics, Univ. Hospital, Rijnsburgerweg 10, 233AA Leyden, Netherlands
Weiden, P. L., Univ. of Washington School of Medicine, Seattle, WA. 98195, USA
Wernet, P., Histokompatibilitätslabor, Medizinische Klinik, Otfried-Müller Str., 7400 Tübingen, West-Germany
Wharam, M. D., Oncology Center, The John Hopkins Univ. School of Medicine, Baltimore, MD. 21205, USA
Wottge, H.-U., Hygiene-Inst. der Univ., Brunswikerstr. 2–6, 2300 Kiel, West-Germany
Zander, A. R., Anderson Hospital and Tumor Inst., 6723 Bertner, Houston, TX. 77030, USA.

Preface

Bone marrow transplantation, the goal which integrates hematologists, immunologists, geneticists, oncologists and specialists of several other fields, has overcome its state of stagnation in recent years. Clinically as well as experimentally new approaches to old problems and new conclusions from recent findings proliferate: bone marrow transplantation in leukemic remission, bone marrow growth in cell culture, bone marrow manipulation with antisera, bone marrow differentiation in histoincompatible hosts, immunosuppression with partial body irradiation to cite just a few. These and other new developments were discussed by experts from 12 countries in and outside the European Community during an international seminar held on March 8–10, 1979 by the "Institut für Hämatologie, GSF", under the auspices of the European Communities.

The editors thank both the contributors to this symposium, who made it a successful meeting and submitted their manuscripts punctually, and the publishers, who have provided a volume of high quality in good time. They are also grateful for the valuable cooperation from numerous colleagues at the Institut für Hämatologie.

Munich, February 1980

S. Thierfelder
H. Rodt
H. J. Kolb

1 Experimental Bone Marrow Transplantation in Rodents

Immune Responses and Tolerance to Donor Tissue in Nude Mice Grafted with an Allogeneic Thymus

Berenice Kindred

A. Reconstitution of Nude Mice with Cell Suspensions

By injecting nude mice with lymphoid cell suspensions from normal mice it is possible to show that a lasting restoration of immune responsiveness occurs only with cell suspensions which contain mature, H-2 compatible T cells. Neonatal thymus cells whether congenic or allogeneic are not effective [12] nor are bone marrow or fetal liver cells [9]. A comparison of the anti-SRBC response after injection of adult CBA, C57BL/6, BALB/c and C57BL/6 × BALB/c F_1 cells into BALB/c-nu is shown in Table 1. Consistent restoration is shown only when the injected spleen or thymus cells are H-2 compatible. BALB/c-Ig^b cells which differ from BALB/c at an allotype locus are able to restore but BALB/c-H-2^b are not. The situation with F_1 cells is peculiar. These are only sometimes able to restore and no explanation can, at present, be offered for this.

Table 1. Number of BALB/c-nu showing a secondary response to SRBC after injection of normal thymus, spleen or lymph node cells from donors of different strains

Donor	Thymus	Spleen	Lymphnode
C57BL/6	0/13	0/6	ND[a]
CBA	0/2	0/4	ND
BALB/c	12/12	10/10	10/10
BALB/c-Ig^b	25/28	10/11	5/9
BALB/c-H-2^b	0/7	ND	ND
BALB/c × C57BL/6	4/16	0/8	ND

[a] Not done

B. Reconstitution with Neonatal Thymus Grafts

When whole, neonatal thymus grafts are used rather than cell suspensions, nude mice can respond to antigens such as SRBC or the bacteriophage T4 regardless of the strain of the thymus donor (Table 2). The reconstitution is not due to cells transferred with the graft since allogeneic thymus cells do not restore. It appears that nude mice have precursors which can enter the grafted thymus, differentiate and repopulate the periphery. BALB/c and AKR differ for the T cell marker, Thy-1 and by grafting neonatal AKR thymuses into BALB/c-nu it could be shown that the thymocyte population changed with time from AKR type to BALB/c type [11].

For about 10 days after grafting the thymocytes were almost entirely of donor type. At 10 days the thymus graft was very small and, histologically,

Donor	H-2 Type	Number	Mean[a]
A	a	14	3.8
DBA/2	d	10	4.5
C57BL/6	b	9	4.6
BALB/c	d	14	5.7
RIII	r	3	6.0
CBA	k	4	6.6
DBA/1	q	9	7.1

[a] Mean Log^{-2} haemagglutination response of 2-mercapto-ethanol treated sera

Table 2. Haemagglutinin response to SRBC in BALB/c-nu 5 weeks after grafting with whole neonatal thymuses from donors of different strains

showed ill-defined lobular structure and poor cortico-medullary differentiation. Between 12 and 26 days AKR cells disappeared and the thymus became entirely populated with BALB/c type cells. During this time the grafts showed marked lymphoid proliferation and a very thick cortex. By 30 days the graft had a fairly normal appearance [6]. T cells of both host and donor type, begin to appear in the spleen and lymph nodes at about 15 days. Donor type cells remain few but host type cells increase in numbers till they constitute about 50% of spleen and lymph node cells at 50 days after grafting [11].

Immune functions which are reconstituted after grafting include response to T cell mitogens [7], helper cell activity [5, 7, 13], third party graft rejection [3, 7, 13] and in vitro cytotoxic response to third party allogeneic cells [1]. It is, however, not clear whether cytotoxic cells capable of killing virus infected cells are generated.

I. Tolerance to Thymus Donor Antigens

In vitro: Figure 1 compares the MLR (mixed lymphocyte reaction) of individual BALB/c-nu mice grafted with either a BALB/c ($H\text{-}2^d$) or C57BL/6 ($H\text{-}2^b$) neonatal thymus and tested with AKR ($H\text{-}2^k$) and C57BL/6 stimulator cells. The mice bearing a BALB/c thymus respond fairly well to both but the mice bearing a C57BL/6 thymus show a marked reduction in the response to C57BL/6 cells.

A similar reduction in the specific response to thymus donor antigens has been demonstrated by Engers et al. [1] using an in vitro cytotoxic cell assay.

In vivo: Rejection of thymus donor and third party skin grafts is shown in Figure 2. BALB/c-nu with either BALB/c or C57BL/6 thymus grafts reject CBA skin and show accelerated rejection of secondary skin grafts. BALB/c BALB/c-nu (BALB/c-nu with a BALB/c thymus graft) show a similar pattern of rejection of C57BL/6 skin but C57 BALB/c-nu are strikingly different. They frequently accept first grafts of C57BL/6 skin and always accept second grafts. This is characteristic of weak graft rejection as shown by Flaherty and Bennett [2].

A second tissue graft to be considered is the thymus graft itself. The thymus epithelium retains the antigens of its own strain [10] but is nevertheless not rejected. Figure 3 shows sections of BALB/c and C57BL/6 thymus grafts from an experiment in which BALB/c-nu were grafted with a neonatal C57BL/6 thymus and 2 months later with a BALB/c thymus. The mice were slain 2 months after the

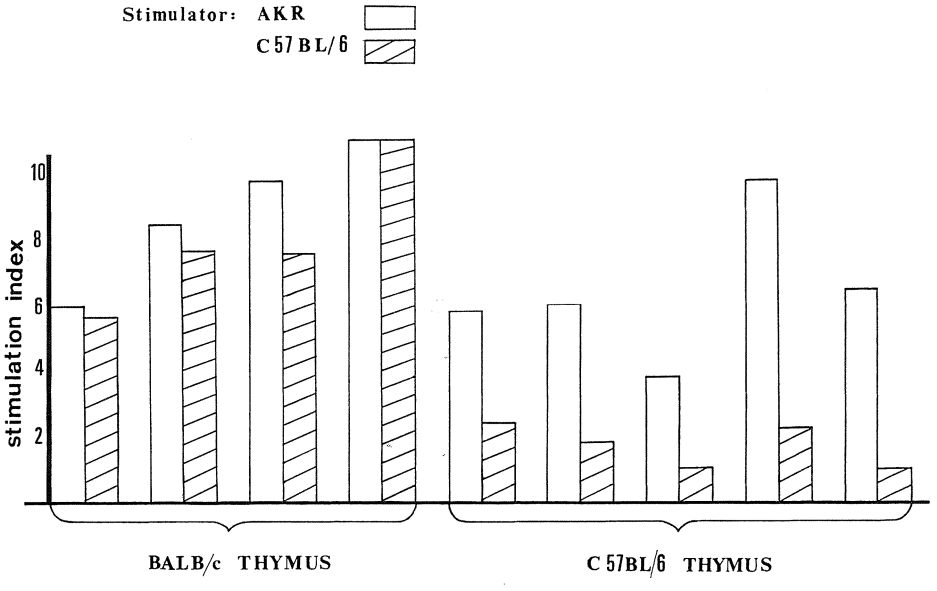


Fig. 1. Comparison of the response to AKR (H-2^k) and C57BL/6 (H-2^b) cells by BALB/c-nu with BALB/c or C57BL/6 neonatal thymus grafts. The response is given as stimulation index (^{3}H incorporation by responder + irradiated AKR or C57BL/6/^{3}H incorporation by responder + irradiated self)

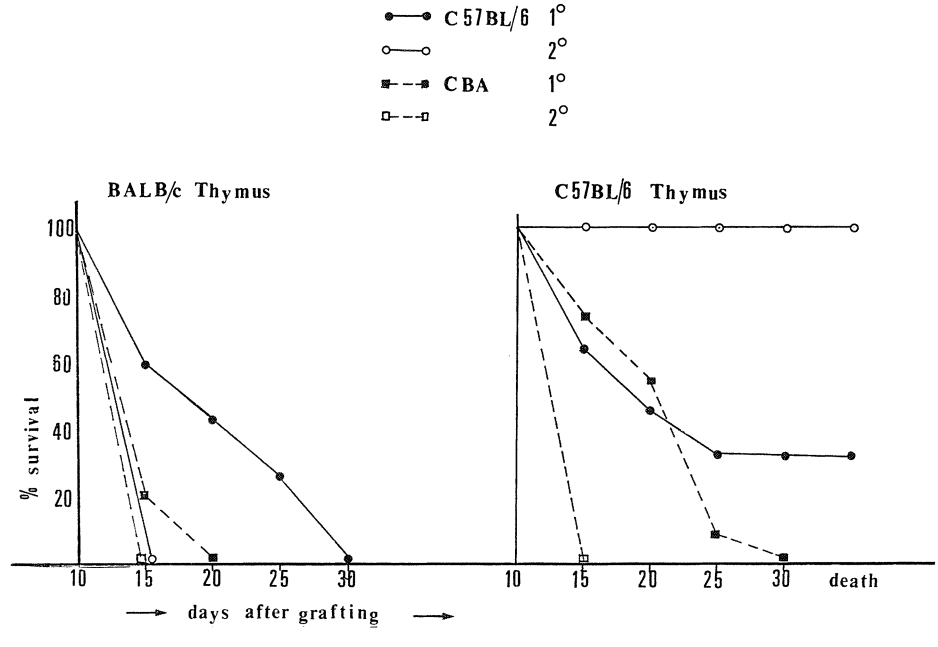


Fig. 2. Cumulative survival of CBA or C57BL/6 skin grafts on BALB/c-nu bearing BALB/c or C57BL/6 thymus grafts

second graft was made and histological preparations were made by Dr. B. Sordat, Lausanne. In general the histological appearance of the BALB/c grafts was closer to normal than that of the C57BL/6 grafts but as shown in Figure 3, viable, fairly normal looking C57BL/6 grafts could be demonstrated even in these mice which had also been grafted with a BALB/c thymus. The most frequent abnormality in the C57BL/6 grafts was the presence of unusual numbers of polymorphonuclear cells.

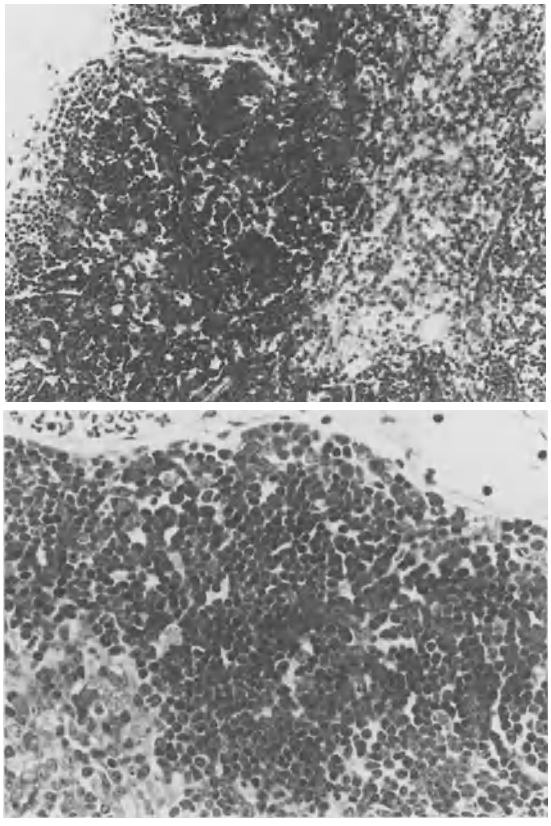


Fig. 3. Section of BALB/c and C57BL/6 thymuses 2 months and 4 months respectively, after grafting into BALB/c-nu mice. *a* Giemsa. 350×, *b* Giemsa. 750×

In all these tests the mice showed strong, but not complete, tolerance to the thymus donor antigens as compared to the response to congenic BALB/c cells. There were also strain differences in the degree of tolerance. BALB/-nu with CBA (H-2^k) thymuses showed good tolerance of CBA tissues while BALB/c-nu with AKR (H-2^k) thymus grafts were poorer and sometimes rejected second grafts of AKR skin.

In this system the unreconstituted nude is incapable of rejecting tissue grafts and the neonatal thymus cells are also incapable of reaction against the host. As the host cells develop they are partially tolerant of the thymus donor antigens and the thymus cells which are transferred with the thymus do not appear to cause a graft versus host reaction although some to migrate out of the graft. However,

Table 3. Survival of BALB/c-nu mice bearing thymus grafts from different donor strains

Thymus donor	H-2 type	Number	Survival	
			1 Month	2 Months
BALB/c	H-2^d	85	75 (88.2%)	69 (81.2%)
C57BL/6	H-2^b	208	171 (82.2%)	128 (61.5%)
A	H-2^a	45	38 (84.4%)	22 (48.9%)
CBA	H-2^k	44	37 (84.1%)	31 (70.5%)
DBA/2	H-2^d	22	ND[a]	10 (45.5%)
DBA/1	H-2^q	20	ND[a]	9 (45.0%)
AKR	H-2^k	19	15 (78.9%)	11 (57.9%)
B10.D2	H-2^d	16	ND[a]	7 (43.8%)

[a] Not done. Deaths in these groups were not regularly recorded during the first month

a comparison, shown in Table 3, of the survival of BALB/c-nu with thymus grafts from different strains shows that survival is best in BALB/c-nu with BALB/c thymus grafts. These data have been collected from many experiments so that they are likely to be influenced by diseases which might occur at some particular time as well as by the treatment e.g. skin grafting to which the animals have been subjected. Nevertheless it is interesting that H-2 compatible DBA/2 and B10.D2 grafts and also H-2D end compatible A grafts to not, in general, permit better survival than H-2 incompatible grafts.

II. Suppressor Cells in Determining Tolerance

The obvious mechanism to consider is elimination, within the thymus, of cells with the potential to react against the antigens of the thymus. However the possibility of peripheral suppression must also be considered. Many attempts were made to demonstrate suppression, either by addition of cells from C57 BALB/c-nu to an MLR or by transferring such cells together with competent cells, to other BALB/c-nu but only one experiment produced positive evidence for suppression. The results of this experiment are shown in Table 4. Although the increase in survival time of CBA skin is small, the range of rejection times of mice injected with C57 nu spleen cells alone, compared with C57 nu + CBA nu spleen cells do not overlap. The increase in survival time of C57BL/6 skin grafts is striking and the C57 nu cells are clearly capable of opposing rejection of C57BL/6 skin by CBA nu cells. However in no case were such cells capable of suppressing the activity of normal BALB/c cells [8]. Therefore it seems probable

Donor	Mean rejection time (range)	
	C57BL/6	CBA
CBA nu	27.3 (18–32)	60+
C57 nu	60+	21.6 (14–23)
CBA nu+ C57 nu	43.5 (42–45)	25.0 (23–27)

Table 4. Rejection times for C57BL/6 and CBA skin grafts by BALB/c-nu recipients of spleen cells from C57 BALB/c-nu or CBA BALB/c-nu donors

that peripheral suppression plays a part in determining tolerance although it is probably not the main part.

III. Role of the Thymus in Determining Tolerance

If an animal has precursor cells potentially capable of recognising all the antigens of the species and the expression of these is selected in the thymus [4], BALB/c cells which differentiate in a C57BL/6 thymus should recognise C57BL/6 as self, but should retain the ability to react against BALB/c. Yet in the mice described here where BALB/c cells have differentiated in a C57BL/6 thymus and operate in a BALB/c environment there is weak but detectable reactivity against C57BL/6 but not against BALB/c. This suggests that the thymus is not entirely responsible for the avoidance of self recognition. It is possible that there is some degree of genetic determination before the procursors reach the thymus or that the environment in which the T cells operate is also important in determination of self tolerance.

References

1. Engers, H. D., Sordat, B., Merenda, C.: Functional reconstitution of nude mice. Generation of cytotoxic T lymphocyte activity in vitro using spleen cells from thymus-grafted nude mice. In press (1979)
2. Flaherty, L., Bennett, D.: Histoincompatibilities found between congenic strains which differ at loci determining differentiation antigens. Transplantation *16,* 505 (1973)
3. Isaak, D. D.: Fate of skin grafts from different inbred strains on nude mice bearing allogeneic thymus grafts. J. reticuloendothel. Soc. *23,* 231 (1978)
4. Jerne, N. K.: The somatic generation of immune recognition. Eur. J. Immunol. *1,* 1 (1971)
5. Jutila, J. W., Reed, N. D., Isaak, D. D.: Studies on the immune response of congenitally athymic (nude) mice. Birth Defects, Original Article Series XI, 522 (1975)
6. Kindred, B.: The nude mouse in studying T cell differentiation. In: The nude mouse in experimental and clinical research. Fogh and Giovanella (eds.), p. 111. New York: Academic Press 1978
7. Kindred, B., Loor, F.: Activity of host-derived T cells which differentiate in nude mice grafted with co-isogenic or allogeneic thymuses. J. Exp. Med. *139,* 1215 (1974)
8. Kindred, B., Sordat, B.: Lymphocytes which differentiate in an allogeneic thymus. II. Evidence for both central and peripheral mechanisms in tolerance to donor strain tissues. Eur. J. Immunol. *7,* 437 (1977)
9. Kindred, B., Weiler, E.: The response to SRBC by nude mice injected with lymphoid cells other than thymus cells. J. Immunol. *109,* 382 (1972)
10. Loor, F., Hägg, L.-B.: T-cell restoration of nude mice. Long term effects of neonatal thymus grafts. 2nd. Internat. Workshop on Nude Mice, p. 233. Tokyo: University of Tokyo Press, 1977
11. Loor, F., Kindred, B.: Differentiation of T-cell precursors in nude mice demonstrated by immunofluorescence of T-cell membrane markers. J. Exp. Med. *138,* 1044 (1973)
12. Pierpaoli, W.: Inability of thymus cells from newborn donors to restore transplantation immunity in athymic mice. Immunology *29,* 465 (1975)
13. Pritchard, H., Micklem, H. S.: Immune response in congenitally thymus-less mice. Clin. Exp. Immunol. *10,* 151 (1972)

Discussion

Bortin: I wonder if you have investigated grafting either syngeneic or allogeneic thymus or thymus cells in a millipore filter chamber.
Kindred: I have used a whole neonatal thymus – syngeneic or allogeneic – in a millipore chamber and was not able to demonstrate any effect whatever. There was no response to T dependent antigens.
Simmonsen: How do you account for the fact that your first graft of C57BL was rejected while your secondary graft of C57BL was tolerated on BALBc mice which were reconstituted with neonatal C57BL thymus. Do you think this was due to suppressor cells?
Kindred: It is a rejection pattern as found in Ly or other weak histoincompatibility. It is possible that it was a response and later tolerance to a skin specific antigen which was not expressed in the thymus, though I don't really like that explanation. It may be suppressor cells.
Thierfelder: Are you sure that your nude mice are 100% immuno-incompetent. Mightn't there be a very minor T-cell population responsible for this partial intolerance?
Kindred: There are about 2–3% theta positive cells in the nude mouse, but with regard to skin graft rejection or a secondary response to antigens like sheep erythrocytes we have never been able to show any function for these theta positive cells. The only function that I know of that has been claimed was a response to flagella.

Transplantation of Cultured Hemopoietic Stem Cells

T. M. Dexter and E. Spooncer

A. Introduction

The hemopoietic system contains a variety of cells with differing capacities for self-renewal, differentiation and maturation, resulting ultimately in the production of the mature blood elements. The earliest, functionally recognisable cell is the spleen colony forming cell or CFU-S, described by Till and McCulloch (1961), which possesses extensive self-renewal ability and pluripotentiality (Metcalf and Moore, 1971, Review). From the CFU-S are derived the various precursor cells ie. committed in pathway of differentiation but retaining extensive proliferative ability, and in vitro systems exist for the clonal proliferation of most of these populations. Granulocyte precursor cells (CFU-C) are recognised by their ability to form colonies containing granulocytes and macrophages, in soft gel media, in the presence of appropriate colony-stimulating factors (CSF) (Bradley and Metcalf, 1966; Pluznik and Sachs, 1966), erythroid precursor cells can be functionally separated into two or more compartments, reflecting their in vitro proliferative ability and their response to erythropoietin and other factors (Stephenson et al., 1971; McLeod et al., 1974; Heath et al., 1976; Iscove, 1978), and include the BFU-E and CFU-E. Furthermore, megakaryocyte progenitor cells can also be induced to undergo clonal proliferation in vitro (Metcalf et al., 1975a) and in the presence of suitable mitogens and growth promoting factors B and T lymphocyte colony formation can also be induced (Metcalf et al., 1975b; Sredni et al., 1976). While these short-term in vitro systems are obviously useful for characterising the colony forming cells (and stimulating factors necessary for their development) they possess the disadvantage of being "running-down" systems – where proliferative activity is rapidly lost.

Recently, we have developed a culture system where stem cell proliferation and differentiation can be maintained in vitro for several months (Dexter et al., 1977a). The maintenance of in vitro hemopoiesis seems to depend upon the prior establishment of an adherent layer of bone marrow cells which we speculate provides the inductive microenvironment necessary for stem cell proliferation and subsequent differentiation to occur. In this communication, the essential characteristics of the culture system are discussed and their relevance to the study of hemopoietic dysplasias and possible usefulness for transplantation purposes is described.

B. Establishing the Cultures

The methods have been described in detail elsewhere (Dexter and Testa, 1977; Dexter et al. 1977a). Briefly, the contents of a mouse femur are flushed into glass or plastic tissue culture flasks (approx. 25 cm^2 growing area) containing 10 ml. Fischer's medium supplemented with 20% horse serum (Flow Laboratories) and antibiotics. No attempt is made to obtain a single cell suspension. The cultures are incubated at 33° C (Dexter et al., 1977a) in air+5% CO_2 and fed weekly by removal of half (5 ml) the growth medium and addition of fresh. Over a 2–3 week period an adherent layer becomes established containing a variety of cell types (probably representative of marrow stromal elements) and the cultures are then fed as before with 5 ml of medium containing 5×10^6–10^7 freshly isolated syngeneic or allogeneic marrow cells. These constitute the cultures proper and are maintained on an appropriate feeding regime (normally weekly demi-depopulation). The growth medium removed contains cells in suspension which after washing and dilution can be assayed for CFU-S or the variety of progenitor cells. In this way we can monitor events occurring in individual cultures over several months.

Note: We have previously reported that only specific batches of horse serum would facilitate the growth of the adherent population and subsequent stem cell maintenance. Recently however, Greenberger (1978) has shown that most serum batches will support stem cell proliferation, providing the cultures are supplemented with 10^{-6} or 10^{-7}M hydrocortisone. We have confirmed these observations.

C. Proliferation and Differentiation of Stem Cells

Total non-adherent cell production and morphology of the cells produced over several weeks of culture is shown in Table 1. Cell proliferation is obviously

Table 1. Production of non-adherent cells in long term marrow cultures

Weeks cultured	Cell count	Morphology %						
		Granulocytes			Macrophage	Lymph	Eryth	Mega
	($x10^5$)	Blasts	Early	Late				
1	49.4	16	14	55	12	0	0	+
3	20.6	9	26	53	12	0	0	+
5	33.4	12	22	60	0	0	0	+
7	88.4	8	6	86	0	0	0	+
10	44.0	6	6	82	2	0	0	+
13	32.4	4	12	80	4	0	0	+
15	15.8	8	9	75	8	0	0	+
17	11.4	6	20	60	14	0	0	+

In medium supplemented with 10^{-6}M hydrocortisone; Lymph=lymphoid cells; Eryth=erythroid cells; Mega=megakaryocytes

occurring for several months – taking into account the weekly depopulation through feeding – with an approximate doubling weekly. During this time, the adherent layer cellularity remains fairly constant. The majority of cells produced consist of granulocytes at all stages of maturation, with a predominance of mature neutrophils. These are functionally more mature cells than those produced in soft agar cultures (Williams et al. 1977). Megakaryocytes are also being produced, but the cultures contain no morphologically recognisable erythroid cells or lymphocytes.

The production of stem cells (CFU-S) and granulocyte precursor cells (CFU-C) is shown in Table 2. There is extensive proliferation of stem cells for at least 3–4 months. These cells are functionally normal, producing all hemopoietic elements in vivo, and can protect mice from potentially lethal irradiation. Similarly, the CFU-C will form granulocyte/macrophage colonies only in the presence of CSF.

Table 2. Production of CFU-S and CFU-C in long-term cultures (non-adherent cells).

Weeks cultured	Non-adherent cells	
	Total CFU-S	Total CFU-C
1[a]	1,335	17,420
3	1,100	ND
5	780	19,200
7	567	11,000
8	945	ND
10	720	ND
12	420	8,000
15	380	6,200

ND = Not done
[a] CFU-S Input = 3,000

In previous work (Testa and Dexter, 1977; Gregory and Eaves, 1978) it has been shown also that long-term cultures will support the production of the most immature erythoid cell series (the BFU-E) but not CFU-E and that megakaryocyte precursor cells are being formed (Williams et al., 1978). Finally, while no mature B or T lymphocytes are present (Dexter et al., 1978), recent evidence suggests the presence of stem cells which are restricted to lymphoid differentiation (ie. can produce T and B cells but not myeloid cells) and a population of cells which has only T-cell potential (? Pre-T-cells) (Phillips, R. B. and Jones, E. V., personal communication 1978).

Consequently, the system embraces proliferation of stem cells and a concomitant differentiation into all the components of the haemopoietic system.

D. Role of the Adherent Layer in in Vitro Hemopoiesis

This has been discussed in detail elsewhere (Dexter et al., 1977a, b; Dexter et al., 1978; Allen and Dexter, 1976; Dexter and Moore, 1977) and may be summarised as follows:

1. The adherent layer, consists of endothelial cells, macrophages and fat cells, often forming a complex multilayer. The presence of fat cells seems particularly important for sustained granulopoiesis and stem cell maintenance.
2. Stem cells and immature granulocytes can be found lodged within the adherent layer, where cellular interactions are obviously occurring. It is not unreasonable to suppose that both the stimulus for proliferation and commitment for differentiation occurs as a result of local cell/cell interactions. Furthermore the adherent layer is a major *site* of stem cell production.
3. Adherent layers derived from S1/S1^d mice (with a genetically determined, defective, haemopoietic stroma), do not support the proliferation of stem cells.

E. Allogeneic Bone Marrow Cultures

One of the problems to concern us was whether an adherent layer derived from one mouse strain was able to reject, or inhibit the growth of, marrow cells from an allogeneic or semi-allogeneic donor (much in the same way that heavily irradiated mice can reject bone marrow cells from allogeneic or semi-allogeneic donors) (Cudkowicz and Stimpfling, 1964; Lotzova and Cudkowicz, 1973). We have found that irrespective of the origin of the adherent layer (ie. allogeneic or semi-allogeneic) it will support the proliferation and differentiation of stem cells. For example, DBA/2 bone marrow cells (H-2^d) will sustain the proliferation of CFU-S from C57B1/6 mice (H-2^b) and vice versa (Dexter and Spooncer, 1978). Other strain combinations are equally successful (Dexter et al., 1977c) and we have been able to maintain three or even four component stem cell chimeras for several weeks. The importance of this, of course, is in human studies, where repeated marrow aspirations from a single donor may not be possible, and where allogeneic combinations may have to be exploited.

F. The Use of Long-term Cultures for Studying Hemopoietic Dysplasias

Given a situation where there is defective haemopoiesis leading to cytopenia, the question arises as to whether this is due to a malfunction at the level of proliferation or differentiation of haemopoietic cells or due to an intrinsic defect in the capacity of the haemopoietic inductive environment (the stroma) to support haemopoiesis. This has important implications in the therapy of such cytopenias, since the natural course for a stem cell defect is marrow transplantation while for a stromal defect an understanding (and use) of the factors controlling haemopoietic cell populations is essential. Marrow transplantation in the latter case would probably be ineffective in the long-term.

Our studies with genetically anemic mice have already shown the potential of long-term cultures in studying these situations (Dexter and Moore, 1977). For example, S1/S1^d mice, which have a defective stroma in vivo, produce adherent layers which are unable to support stem cell replication. S1/S1^d stem cells, however, are normal and will undergo proliferation when added to a normal (wild type) adherent layer. W/W^v mice, on the other hand, possess an intrinsic defect in

the stem cell (although the inductive environment is normal in such mice) such that W/W^v marrow cells will not undergo sustained haemopoiesis when inoculated onto normal adherent layers. Consequently, haemopoiesis rapidly declines when (defective) $S1/S1^d$ adherent layers are inoculated with W/W^v stem cells but is *sustained* when *W/W^v adherent* layers are recharged with *$S1/S1^d$ stem cells.*

We have used this system to study the haemopoietic defect seen in mice which have been given 4×450 rad x-rays – at approximately three week intervals. In such mice, CFU-S and CFU-C recover to a value of only 10% that seen in control, non-irradiated animals (Hendry et al., 1974). When adherent layers were established from such mice, and re-charged with normal stem cells, haemopoiesis rapidly declined ie the environment is defective. When the stem cells from the irradiated mice were added to *normal* adherent layers, haemopoiesis similarly declined (Dexter et al., 1979) ie. the defective haemopoiesis observed in the irradiated mice is probably due to a dual effect of stem cells and the inductive environment.

These examples serve to illustrate the potential of the system for the study of human haemopoietic aplasias.

G. Transplantation of Cultured Stem Cells

One of the major problems in marrow transplantation is the development of graft-versus-host disease (G-v-H disease). This is elicited by contaminating T-lymphocytes present in the bone marrow cells transplanted (von Boemer et al., 1976) – which presumably undergo amplification and reaction against host tissue antigens, resulting in extensive tissue damage and death. As discussed previously, the long-term cultures sustain stem cell proliferation, production of immature committed progenitor cells and granulocyte and megakaryocyte maturation. No lymphopoiesis is seen, however, suggesting that the long term cultures may represent a unique source of cells for marrow transplantation.

When normal C57B1/6 bone marrow cells were injected into irradiated BDF_1 (C57Bl/6 × DBA/2) hybrid mice, 90% of the animals had died by seven weeks – all with evidence of extensive G-v-H disease. However, when *cultured* C57Bl/6 bone marrow cells were injected, 80% of the animals survived for periods in excess of 6 months – with no evidence of G-v-H disease (Fig. 1) (Dexter and Spooncer, 1978). Examination of such animals showed that in 17/18 of animals studies, the haemopoietic system was reconstituted with cells of donor origin (the remaining animal showed a mixture of donor and host cells – presumably representing a re-emergence of a few surviving host stem cells following the irradiation). The reconstituted mice possessed normal numbers of B and T lymphocytes and were immunocompetent, showing no development of runt disease. These data conform with those of other workers (von Boehmer et al., 1976; Muller-Rucholtz et al., 1975) who showed an abrogation of G-v-H potential following removal of T-lymphocytes by immunological procedures or by agglutination and removal of stem cells with specific lectins (Reisner et al., 1978).

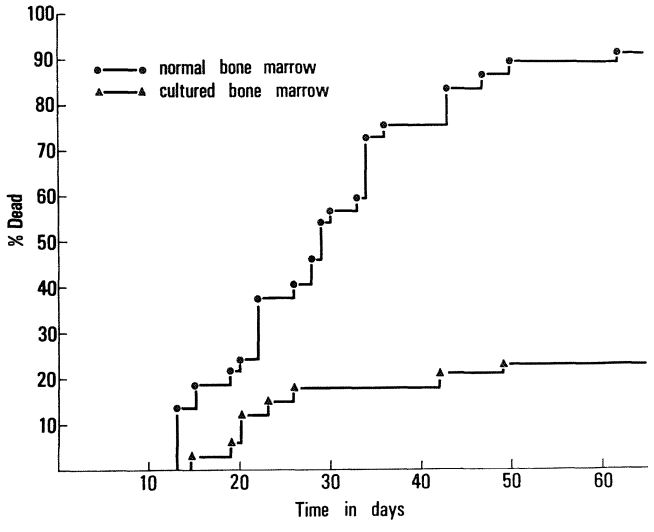


Fig. 1. Death due to graft-versus-host disease in irradiated BDF_1 mice injected with semi-allogeneic C57Bl/6. Normal bone marrow or cultured bone marrow cells

Since the evidence indicates that some HLA homology must exist between recipient and donor for successful marrow transplantation and restoration of immunocompetence (Zinkernagel, 1978 – Review) it follows that the longterm cultures will not provide a situation of a "universal donor". However, the extent of homology required for restoration of lymphocyte function is not really known – and the restriction specificity in inbred mouse strains may not apply in outbred human populations. Moreover, at the present, 80% of close HLA matched human marrow transplantations show evidence of G-v-H – (Thomas, 1978) and long-term marrow cultures may obviate this effect, simply by selective depletion of lymphoid cells.

Finally, the overall output of stem cells in the long-term cultures can be several fold higher than the original input of stem cells – and cryopreservation should allow storage of the stem cells produced, maybe for transplantation at a later date.

Hopefully, recent work by several groups in establishing and maintaining long-term human marrow cultures will realise some of these potentials.

Acknowledgements

During the course of this work we have had many stimulating discussions with Professor L. G. Lajtha and other colleagues in the hematology section, to whom we are indebted. TMD is a Cancer Research Campaign Fellow. This work was supported by the Medical Research Council and the Cancer Research Campaign.

References

Allen, T. D., Dexter, T. M.: Cellular interrelationships during in vitro granulopoiesis. Differentiation *6,* 191–194 (1976)

Boehmer, H. von; Sprent, J., Nabholz, M.: Haemopoietic reconstitution obtained in F_1 hybrids by grafting of parental marrow cells. Transplant. Proc. *8,* 355–358 (1976)

Bradley, T. R., Metcalf, D.: The growth of mouse bone marrow cells in vitro Aust. J. Exp. Biol. Med. Sci. *44,* 287–300 (1966)

Cudkowicz, G., Stimpfling, J. H.: Deficient growth of C57B1 mouse marrow cells transplanted in F_1 hybrid mice. Association with the histocompatibility – 2 locus. Immunology *7,* 291–306 (1964)

Dexter, T. M., Moore, M. A. S.: In vitro duplication and "cure" of haemopoietic defects in genetically anaemic mice. Nature *269,* 412–414 (1977)

Dexter, T. M., Spooncer, E.: Loss of immunoreactivity in long-term bone marrow culture. Nature *275,* 135–136 (1978)

Dexter, T. M., Testa, N. G.: Differentiation and proliferation of haemopoietic cells in culture. In: Prescott, D. M. (ed.), Methods in cell biology, Vol. XIV., pp. 387–405. New York: Academic Press 1976

Dexter, T. M., Allen, T. D., Lajtha, L. G.: Conditions controlling the proliferation of haemopoietic stem cells in vitro. J. Cell. Physiol. *91,* 335–344 (1977a)

Dexter, T. M., Wright, E. G., Krizsa, F., Lajtha, L. G.: Regulation of haemopoietic stem cell proliferation in long term bone marrow cultures. Biomedicine *27,* 344–349 (1977b)

Dexter, T. M., Moore, M. A. S., Sheridan, A. P. C.: Maintenance of haemopoietic stem cells and production of differentiated progeny in allogeneic and semi-allogeneic bone marrow chimeras in vitro. J. Exp. Med. *145,* 1612–1616 (1977c)

Dexter, T. M., Allen, T. D., Lajtha, L. G., Krizsa, F., Testa, N. G., Moore, M. A. S.: In vitro analysis of self-renewal and commitment of haematopoietic stem cells. In: Differentiation of normal and neoplastic haematopoietic cells. Clarkson, B., Marks, P. A., Till, J. E. (eds.), pp. 63–80. Cold Spring Harbor Laboratory 1978

Dexter, T. M., Schofield, R., Hendry, J., Testa, N. G.: Congenital and induced defects in haemopoietic environments, stem cell proliferation and differentiation. In: Aplastic anemia – pathophysiology and approaches to therapy. Berlin, Heidelberg, New York: Springer 1979

Greenberger, J. S.: Sensitivity of corticosteroid-dependent insulin-resistant lipogenesis in marrow pre-adipocytes of obese-diabetic (db/db) mice. Nature *275,* 752–754 (1978)

Gregory, C. J., Eaves, A. C.: In vitro studies of erythropoietic progenitor cell differentiation. In: Differentiation of normal and neoplastic hematopoietic cells. Clarkson, B., Marks, P. A., Till, J. E. (eds.), pp. 179–192. Cold Spring Harbor Laboratory 1978

Heath, D. S., Axelrad, A. A., McLeod, D. L., Shreeve, M. M.: Separation of the erythropoietin-responsive progenitors BFU-E and CFU-E in mouse bone marrow by unit gravity sedimentation. Blood *47,* 777–792 (1976)

Hendry, J. H., Testa, N. G., Lajtha, L. G.: Effect of repeated doses of X-rays of 14 MeV neutrons on mouse bone marrow. Radiat. Res. *59,* 645–652 (1974)

Iscove, N. N.: Erythropoietin-independent stimulation of early erythropoiesis in adult marrow cultures by conditioned media from lectin stimulated mouse spleen cells. In: Hematopoietic cell differentiation. Golde, D. W., Cline, M. J., Metcalf, D., Fox, C. F. (eds.), pp. 37–52. New York: Academic Press 1978

Lotzova, G., Cudkowicz, G.: Resistance of irradiated F_1 hybrid and allogeneic mice to bone marrow grafts of NZB donors. Transplantation *110,* 791–800 (1973)

McLeod, D. L., Shreeve, M. M., Axelrad, A. A.: Improved plasma culture system for production of erythrocytic colonies in vitro: quantitative assay method for CFU-E. Blood *44,* 517–534 (1974)

Metcalf, D., Moore, M. A. S.: Haemopoietic cells. Amsterdam: North Holland Publishing Co. 1971

Metcalf, D., MacDonald, H. R., Odartchenko, N., Sordat, L. B.: Growth of mouse megakaryocyte colonies in vitro. Proc. Natl. Acad. Sci. USA *72,* 1744–1748 (1975a)

Metcalf, D., Warner, N. L., Nossal, G. J. V., Miller, J. F. A. P., Shortman, K., Rabellino, E.: Growth of B lymphocyte colonies in vitro from mouse lymphoid organs. Nature *255,* 630–632 (1975b)

Muller-Rucholtz, W., Wottge, H. U., Muller-Hermelink, H. K.: Bone marrow transplantation rats across strong histio-compatibility barriers by selective elimination of lymphoid cells in donor marrow. Transplant. Proc. *8,* 537–541 (1976)

Pluznik, D. H., Sachs, L.: The induction of colonies of normal "mast" cells by a substance in conditioned medium. Exp. Cell. Res. *43*, 553–563 (1966)
Reisner, Y., Itzicovitch, L., Meshorer, A., Sharon, N.: Haemopoietic stem cell transplantation using mouse bone marrow and spleen cells fractionated by lectins. Proc. Natl. Acad. Sci. USA *75*, 2933–2936 (1978)
Sredni, B., Kalechman, Y., Michlin, H., Rozenzajn, L. A.: Development of colonies in vitro of mitogen stimulated mouse T-lymphocytes. Nature *259*, 130–132 (1976)
Stephenson, J. R., Axelrad, A. A., McLeod, D. L., Shreeve, M. M.: Induction of colonies of haemoglobin synthesising cells by erythropoietin in vitro. Proc. Natl. Acad. Sci. USA *68*, 1542–1546 (1971)
Testa, N. G., Dexter, T. M.: Long-term production of erythroid precursor cells (BFU) in bone marrow cultures. Differentiation *9*, 193–195 (1977)
Thomas, E. D. L.: Marrow transplantation for acute leukemia. Cancer *42*, 895–900 (1978)
Till, J. E., McCulloch, E. A.: A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. *14*, 213–222 (1961)
Williams, N., Jackson, H., Rabellino, E. M.: Proliferation and differentiation of normal granulopoietic cells in continuous bone marrow cultures. J. Cell. Physiol. *93*, 435–400 (1977)
Williams, N., Jackson, H., Sheridan, A. P. C., Murphy, M. J., Elste, A., Moore, M. A. S.: Regulation of megakaryopoiesis in long-term murine bone marrow cultures. Blood *51*, 254–255 (1978)
Zinkernagel, R. M.: Thymus and lymphohaemopoietic cells: their role in T-cell maturation in selection of T cells, H-2-restriction specificity and in H-2 linked 1r gene control. Immunol. Rev., *42*, 224–270 (1978)

Discussion

van Bekkum: What was the number of bone marrow cells injected and dont you think it strange that you find such an early start of so-called graft-versus-host disease in this mild combination? How do you diagnose gvh?
Dexter: We were injecting about 10^7 cells. Mice with gvh had an enlarged spleen with particular lesions, they dont look at all well.
Simonsen: Were the T and B cells all of donor type?
Dexter: yes
Kubanek: Why do you think you dont get any differentiation to CFU-E from your BFU-E, have you tried to add erythropoetin or change culture conditions like going up with the temperature?
Dexter: The interesting thing is that we get absolutely no differentiation into CFU-E, even in the presence of erythropoetin. We think it is because the bone marrow environment that we have in the cultures imposes a particular differentiation block on those erythroid precursor cells[1].
Slavin: Did you check for chimaerism and find T cells of donor origin? Is gvh a lack of donor type T cells?
Dexter: They were all of donor type. We assume that the absence of gvh is due to a lack of T cells in the cultured marrow cells.

[1] Eliason, J. F., Testa, N. G., Dexter, T. M.: Erythropoietin stimulated erythropoiesis in long-term bone marrow culture. Nature *281*, 382–384 (1979)

Stromal Mechanisms of Bone Marrow: Cloning in Vitro and Retransplantation in Vivo

A. J. Friedenstein

Since the existence of hemopoietic stem cells has been proved experimentally and these cells together with their descendants were found to spread within the hemopoietic system and to form colonies in hemopoietic organs [24], another challenging problem came to light, i.e. the problem of differentiation factors in hemopoiesis. The development of hemopoietic cells is believed to be regulated by local differentiation factors distributed among various hemopoietic organs. They produce a short-distance effect upon hemopoietic cells which largely accounts for different cellular content in various hemopoietic organs. The effect of local differentiation factors is manifested, in particular, during formation of hemopoietic colonies in irradiated recipient-mice. A typical feature of the colonies is high homogeneity of their cellular content as compared to the normal hemopoietic tissue [24]. Morphologically discernible cells of essentially the same type are found in the colonies by the $7–10^{th}$ day. At the same time the colonies are shown to have polypotent stem cells and committed precursors for all the three types of hemopoiesis, as well as B cell precursors [15, 21]. Hence, for example, the development of myeloid precursors (regardless of the degree of their commitment) is inhibited while they are still included in the erythroid colonies whereas the development of erythroid precursors is suppressed in the myeloid colonies. It would be natural to assume that the direction of cell development within the colony depends on the local effects which appear to be either permissive or inhibiting for particular types of differentiation. This assumption is confirmed by the colony development in the spleen containing fragments of transplanted bone marrow tissue [28]. In these conditions 7-day old colonies are often found to be of mixed composition: the part of the colony located on the bone marrow stroma is essentially myeloid while the part located on the splenic stroma is largely erythroid. It is difficult to find any other explanation of the phenomenon except that of a "microgeographic effect". Another relevant example is the so-called block of differentiation of hemopoietic stem cells, i.e. absence of the erythroid precursor production when less than 10% of hemopoietic stem cells survive irradiation [2].

Analysis of individual endogenous spleen colonies showed [4] that erythroid cells are produced in them when the total number of hemopoietic stem cells in the animal is even lower than 10% and when, resulting from partial splenectomy, only a small amount of the splenic tissue is retained. Furthermore, the erythroid cell population does not depend on the number of hemopoietic colonies formed in the spleen nor on the contacts among them. The colony size turned out to be a critical factor: erythroid differentiation occurs only in case the average diameter of the colony exceeds 150 μ. It is a question then of the local rather than general effects produced upon colony cells.

On the whole, the hemopoietic system is believed to produce local regulatory effects on hemopoietic cells through their microenvironment.

The microenvironment is formed by the stroma of blood forming organs rather than by their hemopoietic cell populations. It was confirmed by the results of heterotopic transplantation of hemopoietic organ fragments. Hemopoietic and lymphoid cells are substituted in heterotropic transplantation by repopulating cells of the recipient while stromal cells remain to be of donor origin [6, 8, 14]. This turns out to be sufficient for the established transplants to restitute the same type of hemopoiesis which was characteristic of the grafted organ [1]. Thus, it does not depend on the transfer of hemopoietic donor cells, but on the donor instruction for differentiation which is forced upon recipient hemopoietic cells, populating the territory of a heterotopic transplant.

The nature of the cells transferring the microenvironment and the mechanisms of their effect on hemopoiesis are at present the problem of importance. One of the feasible ways for solving the problem consists in cloning of stromal mechanocytes in vitro [9, 13, 23]. It helps to determine the properties of clonogenic cells and to study the ability of their descendants to form the hemopoietic microenvironment. It has been shown that the strains of stromal mechanocytes arising as a result of in vitro passaging of clonogenic stromal fibroblast descendants form specific hemopoietic territories on retransplantation in vivo [10]. Thus, following transplantation of bone marrow stromal cells under the renal capsule in rabbits a bone marrow organ is formed, whereas after transplantation of splenic stromal cells a lymphoid organ is formed.

Below are our latest results obtained in the study of bone marrow clonogenic stromal cells.

Hemopoietic and lymphoid tissues are easily separated into isolated cells in the course of their light mechanical treatment. Consequent explantation into monolayer cultures may be employed as a method of selective cloning of stromal mechanocytes of these tissues [7, 11, 13, 23]. Clonogenic precursors of mechanocytes (FCFC) adhere to the surface of the culture vessel and actively proliferate in vitro. Owing to their low concentration in hemopoietic tissues, they turn out to be widely separated from each other within the culture by cells of other types. As a result, FCFC descendants form discrete colonies of fibroblasts while the bulk of explanted cells (both hemopoietic and lymphoid) serves as a natural feeder.

Visible colonies of fibroblasts are formed 5 to 10 days after explantation of cells derived from bone marrow, spleen, thymus and lymph nodes of adult mice, rats, guinea pigs, rabbits, dogs and humans [7, 13, 23, 26]. The colony formation starts on the 3–4th day when the colonies consist of a few cells. By the 10th day some colonies grow to a diameter of 0.5–0.8 cm and include several thousands of cells. Between the 5–12th day of cultivation the number of colonies remains the same while the size of many of them increases (Fig. 1).

The cells constituting a colony are typical fibroblasts. Fibrils are generally visible in their cytoplasm and a large nucleolar complex is discernible in the nuclei. Fibroblast colonies usually grow with standard synthetic media (199, Eagle, Fischer) supplemented with 10–20% of the serum and do not require any additional growth-stimulating factors. The formation of colonies occurs only

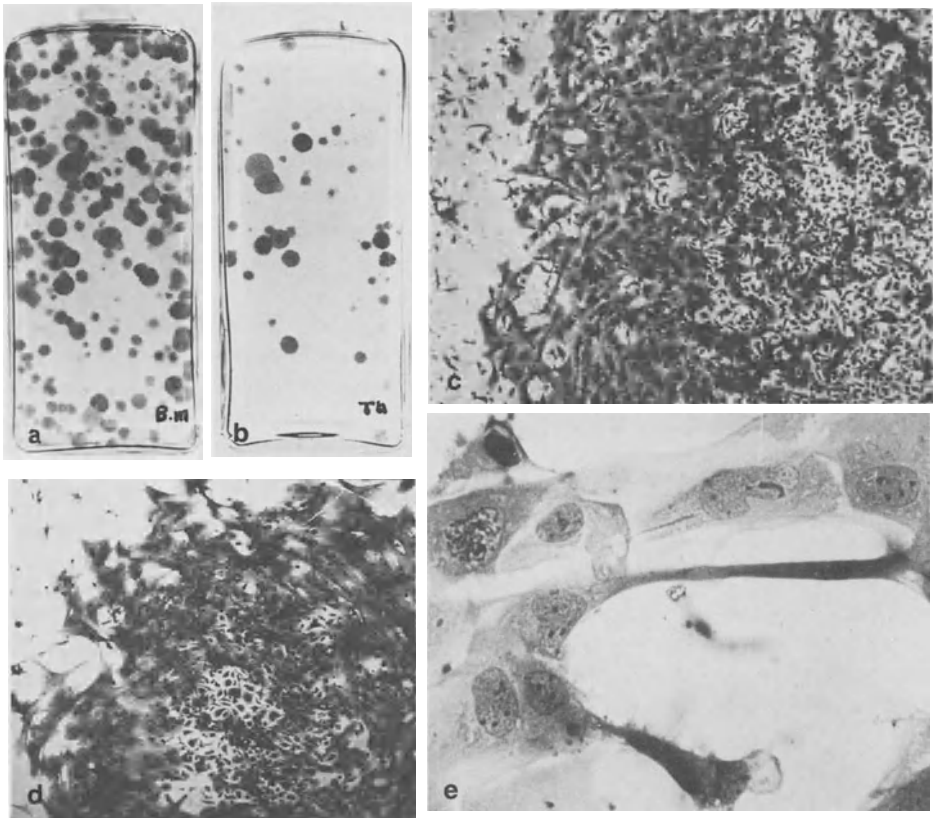


Fig. 1. Fibroblast colonies in monolayer cultures of cells from hemopoietic organs., **a** 12 day culture of guinea pig bone marrow cells; **b** 12 day culture of guinea pig thymus cells; **c** 12 day culture of bone marrow cells from nude mice; **d** 12 day culture of bone marrow cells from CBA mice; **e** 7 day culture of guinea pig bone marrow cells

when the explantation is performed at a certain optimal initial density of cells: in case of excessive number of explanted cells per a surface unit fibroblasts form a monolayer, whereas in case of insufficient density they fail to grow at all. Stable colony-forming efficiency (CFE-F) resulting in a linear correlation between the number of colonies and that of explanted cells is achieved on explantation at the initial density of 10^4–10^5 lymphoid or hemopoietic cells per cm^2. From 5×10^5 to 5×10^6 bone marrow cells exposed to 5,000 rad should be employed as a supplementary feeder if the initial density of explanted cells is lower. CFE-F in culturing a mixture of bone marrow cells from different donors is additive to colony-forming efficiency in separate cultivation of cells from individual donors; in the presence of a standard feeder (irradiated bone marrow cells) the differences of CFE-F characteristic of individual donors are also retained.

All this indicates that an adequate feeder effect, i.e. providing stable CFE-F, results merely from availability of a sufficient amount of bone marrow cells

(including irradiated cells) and is not a priviledge of a particular category of cells whose bone marrow content varies in different donors.

The culture medium composition ensuring maximum CFE-F was selected for bone marrow cells of different species. For human cells and those of guinea pigs, rats, hamsters and rabbits medium 199 was used or the double Eagle medium; for mice cells the Eagle medium supplemented with a nutrient cocktail [25] or the Fischer medium was used. In all cases the medium should contain 10–20% of serum: embryonal calf serum – for cells of guinea pigs, hamsters, rats and mice; homologous serum supplemented with 5% embryonal calf serum – for human cells and those of rabbits.

Besides morphological characteristics there is some additional evidence to confirm that colony-constituting cells are really mechanocytes. The colony cells synthesize fibronectin and collagen (the latter is detected both histochemically and by means of labeled proline inclusion) [13]; these proteins are also secreted into the medium. Contrary to macrophages, fibroblast colony cells have low esterase-I activity and are devoid of receptors for IgG, IgM and complement. On their surface there is a specific fibroblast antigen [16, 17]. It is a protein with electrophoretic motility of α-globulin, which is secreted into the medium. This antigen is not found on macrophages, lymphocytes or hemopoietic cells. In immunofluorescence reactions the anti-fibroblast serum becomes specifically bound to cells, constituting stromal colonies but not to neighbouring macrophages. In the presence of a complement this antiserum exerts a cytotoxic effect upon colony cells, whereas without a complement it causes detachment of colony cells but not of macrophages within 2 hours.

So far very few constant morphological signs have been revealed to differentiate fibroblast colonies regarding their origin from different hemopoietic organs. It is still unclear which type of collagen is synthesized by stromal mechanocytes and whether this sign can be used to differentiate mechanocytes of various hemopoietic organs as well as individual colonies within one culture.

Macrophages are retained in bone marrow cultures of mice for a long time, in contrast to bone marrow cultures of guinea pigs, rabbits and humans. They form a cellular layer between colonies and are also found on the colony surface, above fibroblasts. Macrophages can be eliminated by treating the cultures with antimacrophage serum with a complement.

Fibroblast colonies are cell clones. It means that all fibroblasts constituting a colony are the offspring of one colony-forming cell. Clonal nature of colonies has been ascertained in several ways. It was shown that individual colonies are formed independently of each other: distribution of the number of colonies during explantation of the same number of cells is near to the Poisson value [7]. During explantation, for instance, of 10^6 to 10^5 bone marrow cells in vessels with the surface area of 42 cm^2 the colony-forming efficiency remains unaltered, i.e. there is a linear correlation between the number of colonies and the amount of cells explanted [7]. It implies that a colony-forming unit consists of either one cell or several cells but its size is constant. In fact, a colony-forming unit consists of one cell and colonies are clones. This has been demonstrated by the chromosome analysis of dividing cells in colonies in mixed cultures of male and female guinea pig cells: each of the colonies contained either cells with only XY chromosomes or

with only XX chromosomes [7]. Finally, time-lapse cinematographic observations of living cultures confirmed that each colony is formed as a result of division of one colony-forming cell.

The concentration of colony-forming cells (FCFC) for bone marrow of humans, rabbits, guinea pigs, hamsters, rats and mice was 1–5 per 10^5 cells; for spleen cells of guinea pigs, rabbits and mice it was 1 per 10^6 cells; for thymus cells of guinea pigs, rabbits and mice the FCFC concentration was 1 per 10^6 cells and for guinea pig lymphnode cells it was 1 per 10^7 cells [13, 23]. These include those precursor cells of stromal mechanocytes (fibroblasts) which are capable of forming clones of no less than 50 cells, i.e. are capable of dividing at least 6 times. It remains to be seen whether all categories of stromal precursor cells have this ability and, hence, may be detected by the cloning method. Yet, even for the category of colony-forming precursors the question of efficiency of cloning, i.e. what proportion of clonogenic explanted cells will in fact form colonies, is still open. Evidently, stromal clonogenic cells are highly resistant to the damaging effect of explantation. Indeed, if several hours following explantation the adhered cells are removed from the glass by means of trypsin and are transferred to a new vessel for further cultivation the removed colony-forming cells will produce colonies with almost 100% efficiency. Generally speaking, the CFE-F value shows the concentration of a particular but, possibly, not the only category of fibroblast precursors available among cells of hemopoietic and lymphoid tissues.

Fibroblast colonies in bone marrow cultures of mice may be classified into three types: containing adipose cells (2%); having high activity of alkaline phosphatase (sufficient for the positive reaction by the Gomori method) (60%); and phosphatase negative. The ratio of these three types of colonies does not vary with the age of cultures. As to adipose cells found in fibroblast colonies it is easy to see that they are nothing but transformed fibroblasts.

Cloning enables one to determine some properties of not only cells within a colony but also of initial clonogenic cells (FCFC).

FCFCs from spleen, thymus and bone marrow belong to slowly proliferating cells. Bone marrow of 6- and 14-day old guinea pigs is found to have, respectively, 15% and 2% of labeled FCFCs after 72 hours of labeling with ^{3}H-thymidine. Following a single administration of ^{3}H-thymidine to adult animals all FCFCs remain non-labeled [19]. They are not labeled nor do they die of "suicide" during in vitro incubation with ^{3}H-thymidine of high specific activity [5]. In this respect FCFCs are markedly different from macrophage precursors 40% of which are labeled during such an incubation.

The FCFC content in hemopoietic organs changes with age [27].

Following explantation all FCFCs enter the synthetic period (S) between the 28^{th} and 60^{th} hour but this concerns only the cells which adhered to the surface of the vessel [18].

The mean value of fibroblast generation time in bone marrow colonies is 20 hours [18]. A proliferative pool in colonies depends on the colony size and the culture age. In colonies of less than 30 cells in 4–5 day old cultures it is near 100%. In larger colonies a part of fibroblasts ceases to divide. On the average, a proliferative pool in colonies in 7 and 12 day old cultures is 85% and 65%,

respectively. Fibroblasts that stopped dividing are retained in colonies for 5–7 days [18, 23].

FCFCs from bone marrow and thymus belong to the category of cells readily adhering to the glass surface. The time required for 90% of FCFCs to adhere is 90 minutes, and most of them adhere during the first 30 minutes [9, 22].

The findings concerning radiosensitivity of stromal cell precursors obtained by different methods agree very closely. Radiosensitivity of FCFCs from bone marrow and spleen of guinea pigs and bone marrow of mice determined by the degree of inhibition of colony formation after in vitro irradiation is characterized by a D_0 of 200 rad and an extrapolation number (n) of 1.4 [20, 12, 9, 10, 13]. Human bone marrow FCFCs have a D_0 of 100 rad.

As pointed out above, stromal mechanocytes in colonies have a specific surface antigen which is not found in hemopoietic, lymphoid cells or macrophages. This antigen is present in FCFCs as well and appears to be their surface tissue-specific marker [17].

Typing of FCFCs in heterotopic semi-syngeneic bone marrow transplants and in bone marrow of radiochimeras (carried out by a cytotoxic complement-dependent action of isoantisera) showed that in the former all FCFCs are of donor origin, while in the latter they are those of a recipient [14]. This makes FCFCs different from hemopoietic, lymphoid cells and macrophages whose origin is just the opposite. Clonogenic stromal cells are not substituted by the recipient cells in bone marrow transplants even 14 months later, regardless of total populating of transplants with hemopoietic host cells. Hence, FCFCs appear to be histogenetically independent of hemopoietic cells and are not replenished at the expense of cell migration from outside, for example, from the stroma of other bone marrow areas (otherwise, heterotopic transplants would have displayed a FCFC admixture of recipient origin). FCFCs, therefore, belong to the local bone marrow cell population and are different in this respect from hemopoietic, lymphoid cells and macrophages [13].

Of particular interest is the question of the role played by clonogenic stromal cells in creating hemopoietic territories and in transferring the microenvironment.

The hemopoietic microenvironment (from bone marrow or spleen) is transferred by transplantation of pure diploid fibroblast strains of, respectively, bone marrow or splenic origin [11]. These cell lines are descendants of clonogenic stromal cells. They are obtained as a result of in vitro passaging of several dozens to hundreds of colonies from primary bone marrow or spleen cultures.

The question arises then whether the microenvironment can be transferred by descendants of one stromal clonogenic cell or there should be co-operation of different clones of stromal mechanocytes. Below are presented the results of transplantation of individual stromal fibroblast colonies from bone marrow cultures [3].

Bone marrow cells from adult CBA or (CBA × C57Bl) F_1 mice and outbred guinea pigs were used for cloning of stromal mechanocytes. In mice, bone marrow cells were obtained from femurs; in guinea pigs they were obtained from pelvic bones.

Cellular suspensions were filtered through four layers of nylon mesh prior to explantation in 100 ml Roux vessels (10^5 cells per cm^2), their glass surface covered with a thin layer of collagen gel. Guinea pig cells were cultivated in medium 199 with 20% bovine serum while mouse cells were cultivated in the Fischer medium with 15% bovine serum and 5% embryonal serum. 10^7 bone marrow cells irradiated by 5,000 rad were added as a supplementary feeder. The medium was changed when pH of the culture changed. On the 16–30th day of cultivation fibroblast colonies were cut out and transplanted in vivo. Colonies of mouse fibroblasts were transplanted to syngeneic recipients under the renal capsule between two millipore membranes forming an open sac. Guinea pig fibroblast colonies were transplanted in two ways: 1. a homologous way consisted in intraperitoneal transplantation of colonies in diffusion chambers made of HA filters; 2. an autologous way consisted in placing colonies inside the diaphysis of a homologous femur (free of marrow and exposed to 5,000 rad) which was then implanted into the anterior abdominal muscle.

Control experiments includes implantation of millipore sacs with embryonal fibroblasts and irradiated bone cylinders without fibroblasts or with subcutaneous fibroblasts.

On the 30–90th day the implants were fixed and histologically treated.

Fibroblast colonies were formed in the culture on collagen gel in the same way like in bone marrow cultures on the glass. By the 14–16th day the size of colonies was from 1 to 10 mm in diameter and they contained several hundreds or thousands of cells, respectively. The colony-forming efficiency was about 3 per 10^5 for guinea pig cells and 6 per 10^5 for mouse cells. There was an insignificant admixture of macrophages in the cultures of guinea pig bone marrow cells and the colonies consisted only of fibroblasts, whereas the admixture of macrophages was essential in mouse bone marrow cultures.

The results of individual colony transplantation in diffusion chambers were as follows: 30% of chambers were found to have foci of bone tissue with osteocytes incorporated into the bone matrix; 10% of chambers contained dead bone in which osteocytes and osteoblasts did not survive by the moment of fixation; there were no signs of osteogenesis in the rest of the chambers.

The results of transplantation of fibroblast colonies into the cell-open systems (under the renal capsule or intramuscularly in irradiated bone cylinders) could be summarized as follows. In the control experiments the cavities of irradiated bone cylinders were filled with connective tissue without any sign of osteogenesis or hemopoiesis. The kidney capsule was slightly thickened at the site of control millipore filter transplantation. The same findings, which are not different from the control experiments, were obtained at the site of transplantation of most fibroblast colonies. However, the transfer of about 30% of colonies under the kidney capsule resulted in the development of bone tissue or a bone marrow organ in 1.5–3 months. Half of these cases were bone tissue with osteocytes but devoid of an osteoblast layer and of a bone marrow cavity, or dead bone tissue without osteocytes or osteoblasts. In another 15% of the cases a new bone marrow organ developed. A bone layer covered the marrow cavity formed under the kidney capsule, and hemopoietic cells (about 10^4 per a transplant) included myeloid, erythroid cells and megacaryocytes. The stroma of the bone marrow

cavity had a well-developed system of sinusoids and typical adipose cells. Cell typing in this bone marrow (formed after transplantation of CBA bone marrow fibroblast colonies under the (CBA × C57BL) F_1 kidney capsule) showed that its hemopoietic cells and macrophages are of recipient origin while clonogenic stromal cells are of donor origin. When located within bone cylinders, new bone marrow organs had a round shape and a capsule made of osseous tissue with a well-developed osteoblast layer. The bone marrow cavity contained reticular tissue with myeloid and erythroid cells in proportions typical of bone marrow (Fig. 2).

The number of clones whose transplantation resulted in the formation of a bone marrow organ was about 15% of the total number of transplanted fibroblast colonies. These were large-sized colonies with densely packed cells.

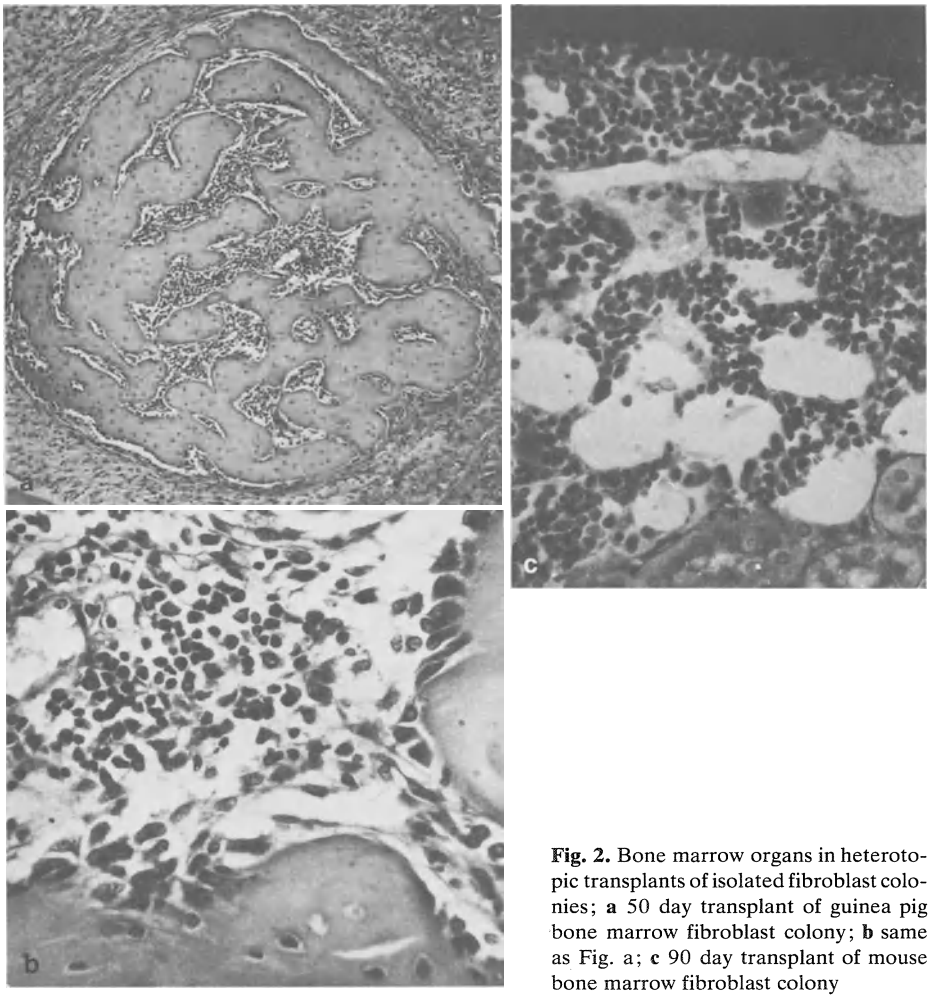


Fig. 2. Bone marrow organs in heterotopic transplants of isolated fibroblast colonies; **a** 50 day transplant of guinea pig bone marrow fibroblast colony; **b** same as Fig. a; **c** 90 day transplant of mouse bone marrow fibroblast colony

These colonies displayed marked alkaline phosphatase activity in mouse bone marrow cultures.

Individual clones of bone marrow mechanocytes, i.e. descendants of one clonogenic stromal cell, are capable, therefore, of not only forming bone but also of transferring the microenvironment for all three differentiation lines of hemopoiesis simultaneous erythroid, myeloid and megacaryocytic. Therewith, one clone of stromal mechanocytes creates a strikingly large hemopoietic territory and a significant amount of bone tissue. However, this feature is not found in all colonies of stromal bone marrow fiberoblasts. Currently available data are not adequate for a clear understanding of the hierarchy among clonogenic cells: those which form non-self-maintaining (dying in the transplants) bone tissue and those which transfer the hemopoietic microenvironment and simultaneously create a bone tissue capable of long-term self-maintenance. It is evident, however, that at least some stromal clonogenic cells are capable of self-maintenance and have such potential for differentiation that the descendants of these cells can do without co-operation with other stromal cells in creating bone marrow hemopoietic microenvironment. Whether these clonogenic fibroblast precursors may be classified as stromal stem cells remains to be seen.

The study of stromal tissue of hemopoietic organs by means of in vitro cloning has just been initiated. Our experience shows that these studies should not be limited to determining only the number of clonogenic stromal cells but should also be based on morphological, histochemical and immunological characteristics of both colony-forming and colony-constituting cells and, whenever feasible, on retransplantation experiments.

Summary

When bone marrow cells from mice, guinea pigs, rabbits, rats, hamsters, dogs and humans are explanted in monolayer cultures, fibroblast colonies are formed. The clonal nature of colonies has been proved by chromosome markers and by time-lapse cinematographic observations. Fibroblast colony-forming cells (FCFC) are highly adhesive, possess fibroblast-specific antigen, lack FC or C receptors, are devoid of non-specific esterase activity. Radiosensitivity of FCFC is characterized by a D_0 of 180 rad and by n of 1.4. FCFC are local non-repopulating cells and are histogenetically independent of hemopoietic cells. In contrast to hemopoietic cells and macrophages, all bone marrow FCFC in radiochimeras are of recipient origin; in heterotopic bone marrow transplants FCFC are of donor origin.

Cells from large fibroblast colonies (containing no less than 10^3 cells in 10–12 day bone marrow cultures) give rise to diploid strains of stromal fibroblasts on passaging in vitro. When transplanted heterotopically these cell strains manifest osteogenic properties and form a bone marrow organ populated by hemopoietic cells.

Heterotopic transplantation of individual bone marrow fibroblast colonies has shown that some of them are able to form bone and to transfer the microenvironment for all lines of differentiation of bone marrow cells simulta-

neously. However, most of the colonies either produced no bone at all or formed the bone tissue with limited self-maintaining capacity; these colonies did not transfer the hematopoietic microenvironment.

References

1. Bernstein, S. E.: Tissue transplantation as an analytic therapeutic tool in hereditary anemias. Am. J. Surg. *119*, 448–451 (1970)
2. Boggs, S., Chervenick, P. A., Boggs, P. D.: The effect of post-irradiation bleeding or endotoxin on proliferation and differentiation of hemopoietic stem cells. Blood *40*, 375–382 (1972)
3. Chailakhyan, R. K., Gerasimov, Yu. V., Friedenstein, A. J.: Stromal mechanocyte clone transfer of bone marrow microenvironment. Bull. Exp. Biol. Med. *86*, 705–707 (1978)
4. DeGowin, R. L., Gibson, D. P.: Hemopoietic colonial response to erythropoietin. Exp. Hematol. *6*, 220–226 (1978)
5. Epichina, S. Y., Latzinik, N. V.: Proliferative activity of clonogenic bone marrow stromal precursor cells. Bull. Exp. Biol. Med. *84*, 55–56 (1976)
6. Friedenstein, A. J., Petrakova, K. V., Kuralesova, A. I., Frolova, G. P.: Heterotopic transplants of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation *6*, 230–247 (1968)
7. Friedenstein, A. J., Chailakhjan, R. K., Lalykina, K. S.: The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet. *3*, 393–403 (1970)
8. Friedenstein, A. J., Kuralesova, A. I.: Osteogenic precursor cells of bone marrow in radiation chimeras. Transplantation *12*, 99–108 (1971)
9. Friedenstein, A. J.: Determined and inducible osteogenic precursor cells. In: Hard tissue growth, repair and remineralization. Ciba Foundation Symposium II (new series), pp. 169–185. North-Holland, Amsterdam: Elsevier 1973
10. Friedenstein, A. J., Chailakhyan, R. K., Latzinik, N. V., Panasyuk, A. F., Keilis-Borok, I. V.: Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation *17*, 331–340 (1974)
11. Friedenstein, A. J., Deriglasiva, U. F., Kulagina, N. N., Panasuk, A. F., Rudakowa, S. F., Luria, E. A., Rudakow, I. A.: Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp. Hematol. *2*, 83–92 (1974)
12. Friedenstein, A. J., Gorskaja, U. F., Kulagina, N. N.: Fibroblast precursors in normal and irradiated mouse hemopoietic organs. Exp. Hematol. *4*, 267–274 (1976)
13. Friedenstein, A. J.: Precursor cells for mechanocytes. Int. Rev. Cytol. *47*, 327–359 (1976)
14. Friedenstein, A. J., Ivanov-Smolenski, A. A., Chajlakjan, R. K., Gorskaya, U. F., Kuralesova, A. I., Latzinik, N. V., Gerasimow, U. W.: Origin of bone marrow stromal mechanocytes in radiochimeras and in heterotopic transplants. Exp. Hematol. *6*, 440–444 (1978)
15. Gregory, C. J., McCulloch, E. A., Till, J. E.: The content of hemopoietic precursors in individual spleen colonies. Probl. Hemat. *10*, 44–48 (1973)
16. Ivanov-Smolenski, A. A.: Characteristics of heteroantisera to stromal fibroblasts. Bull. Exp. Biol. Med. *86*, 454–458 (1978)
17. Ivanov-Smolenski, A. A., Grosheva, A. G.: Heteroantisera to bone marrow stromal mechanocytes. Bull. Exp. Biol. Med. *86*, 451–454 (1978)
18. Keilis-Borok, I. V., Latzinik, N. V., Epichina, S. Y., Friedenstein, A. J.: Dynamics of the formation of fibroblast colonies in monolayer cultures of bone marrow according to H^3thymidine incorporation experiments. Cytologia *13*, 1402–1409 (1971)
19. Keilis-Borok, I. V., Latzinik, N. V., Deriglasova, Y. F.: H^3thymidine incorporation by bone marrow fibroblast precursors. Bull. Exp. Biol. Med. *82*, 971–973 (1972)
20. Kusmenko, G. N., Panasjuk, A. F., Friedenstein, A. J., Kulagina, N. N.: Radiosensitivity of bone marrow cells forming fibroblast colonies in monolayer cultures. Bull. Exp. Biol. Med. *80*, 94–97 (1972)

21. Lala, P. K., Johnson, G. R.: Monoclonal origin of B lymphocytes colony-forming cells in spleen colonies formed by multipotential hemopoietic stem cells. J. Exp. Med. *148,* 1468–1477 (1978)
22. Latzinik, N. V., Epichina, S. Y.: Adhesive properties of fibroblast colony-forming cells from hemopoietic and lymphoid tissues. Bull. Exp. Biol. Med. *81,* 86–89 (1973)
23. Luria, E. A.: Hematopoietic and lymphoid tissues in culture p. 181, New York, London: Plenum Press 1977
24. Metcalf, D., Moore, M. A. S.: Haemopoietic cells p. 550, Amsterdam, London: North-Holland Publishing Comp. 1971
25. Mishell, R. I., Dutton, R. W.: Immunization of dissociated spleen cell cultures from normal mice. J. Exp. Med. *126,* 423–438 (1967)
26. Panasyk, A. F., Luria, E. A., Friedenstein, A. J., Kulagina, N. N., Gerasimov, Y. W., Smirnow, A. N.: Cultures of human bone marrow fibroblast-like cells. Probl. Hemat. *10,* 34–39 (1972)
27. Sidorovich, S. Y., Latzinik, N. V.: Age-related changes of clonogenic stromal precursors in hemopoietic and lymphoid organs. Bull. Exp. Biol. Med. *86,* 96–98 (1978)
28. Wolf, N. S., Trentin, J. J.: Hemopoietic colony studies. J. Exp. Med. *127,* 205–214 (1968)
29. Ivanov-Smolenski et al.: Diploid strains of guinea pig bone marrow and thymus fibroblasts synthesize type I and type III collagen (in press)
30. Moore, M. A. S.: Fibroblasts composing colonies in bone marrow cultures lack factor VIII antigen present in cultured endothelial cells (personal communication)

Graft-Versus-Leukemia: Allosensitization of MHC Compatible Donors Induces Antileukemic Reactivity Without Amplification of Antihost Reactivity

M. M. Bortin, R. L. Truitt, C. Y. Shih, and A. A. Rimm

A. Introduction

Allogeneic marrow transplantation from donors compatible with the hosts at the major histocompatibility complex (MHC) is being used with increasing frequency for patients with forms of leukemia which are refractory to sublethal doses of chemotherapy [5, 22, 34, 35, 40]. The transplanted marrow restores hematopoietic and lymphocyte function after otherwise lethal doses of chemotherapy and radiotherapy. Nonetheless, recurrent leukemia is a major cause of failure in patients treated with "megadoses" of chemoradiotherapy followed by MHC compatible marrow transplantation [5, 22, 34, 35, 40]. There is little convincing evidence, however, that the immunocompetent cells or their progeny in marrow transplants from HLA compatible donors had an adoptive immunotherapeutic effect and caused a graft-versus-leukemia (GVL) reaction [5, 22, 30, 34, 35, 40].

Similarly, in both long-passage and spontaneous T cell acute lymphoblastic leukemia of AKR mice (AKR-L), we found no evidence that transplanted immunocompetent cells from MHC compatible donors had GVL reactivity as measured in a six-day bioassay [4, 7, 12, 23]. On the other hand, using the same bioassay system, cells from MHC *in*compatible donors had highly significant GVL reactivity [4, 12, 23]. Obviously, transplantation of MHC *in*compatible marrow carries with it the very great likelihood of lethal graft-versus-host (GVH) disease. As long ago as 1965, Mathé et al. [27] reported that secondary disease was the major stumbling block to successful application of adoptive immunotherapy. The problem of secondary disease, i.e., GVH disease and the infectious complications associated with the prolonged period of immunodeficiency following allogeneic marrow transplantation, remains as the single most important obstacle preventing successful clinical application of adoptive immunotherapy.

Therefore, the foremost objective of our research and that of others working in the field of adoptive immunotherapy has been to devise strategies to obtain the desired GVL effect while avoiding the undesired GVH disease.

It has been possible to circumvent lethal GVH disease following MHC *in*compatible marrow transplantation in treatment models of acute leukemia by using a transient GVL reaction [3, 4, 6, 10, 12–14, 39] or by use of antibiotic decontamination and protective isolation of the host [36–38]. More recently we attempted to induce GVL reactivity in MHC compatible donor mice without intensification of their GVH reactivity. Immunization of MHC compatible donors with irradiated AKR leukemia (AKR-L_x) cells induced GVL reactivity but was associated with an unacceptable increase in the GVH reactivity of the

donor cells [12]. Immunization of MHC compatible CBA donors with solubilized AKR-L cell surface antigens failed to induce GVL reactivity [11]. Immunization of CBA mice with human T cell acute lymphoblastic leukemia cell lines also failed to induce GVL reactivity (unpublished). Although immunization of CBA donors with LT_1, a Gross-virus induced rat leukemia, generated significant GVL reactivity, the level was low [11]. Incubation of CBA donor cells with xenogeneic immune RNA (from lymphocytes of guinea pigs that had been immunized with AKR-L cells) also failed to induce GVL reactivity [11]. Use of AKR-L_x cells to sensitize CBA cells in vitro in an effort to generate specific cytotoxic effector cells was largely ineffective as measured in cell mediated lympholysis of radiolabeled AKR-L cells [11].

A number of recent reports indicated that genetically inappropriate histocompatibility antigens were found on the cell membrane of chemically induced, virally induced and spontaneous tumors, and that allosensitization of syngeneic hosts induced reactivity against these tumors [1, 2, 16–20, 25, 26, 28, 29, 31, 32, 41, 42]. We now report that allosensitization of CBA (H-2^k) donor mice with lymphoid cells from a variety of healthy unrelated strains of mice induced GVL reactivity against AKR-L in vivo without modification of GVH reactivity.

B. Materials and Methods

Mice. All mice were obtained from the Jackson Laboratory (Bar Harbor, ME), were housed in filter-capped cages, and given autoclaved mouse chow and acidified, chlorinated water ad libitum.
Irradiation. Young AKR mice were exposed to 800 r total body γ-irradiation at a midline tissue dose of approximately 109 r per minute from twin ^{137}Cs sources in a Gammacell-40 (Atomic Energy of Canada, Inc., Ottawa) small animal irradiator. AKR-L cell suspensions used for immunization were exposed to 3,000 r.
Cell Suspensions. Femoral bone marrow, spleen, thymus, and mesenteric lymph nodes were collected, processed into monodispersed cell suspensions, counted in a Cytograf Model 6300A (Ortho Instruments, Westwood, Mass.), and tested for viability using trypan blue dye exclusion. Female AKR retired breeders between five and twelve months of age were examined weekly by palpation; those with splenomegaly plus inguinal and axillary lymphadenopathy were considered to be leukemic. All AKR-L cells came from fresh pools of 15 leukemic AKR spleens. The pooled leukemic spleens were processed into monodispersed cell suspensions, counted, and tested for viability as above.
Immunizations. For specific immunization, CBA mice were given six weekly i.p. injections of 10^7 AKR-L_x spleen cells. For allosensitization, CBA mice were given six weekly i-p. injections of 10^7 mixed spleen and thymus cells from healthy donors. CBA mice were used as donors 7 to 8 days after the last immunization. We found no significant difference in the results whether 10^7 bone marrow plus 10^7 lymph node cells or 2×10^7 spleen cells from the CBA donors were used in the GVL and GVH assays; therefore, no distinction has been made in the presentation of the data and, in several instances, the results have been combined.
Bioassay for GVL Reactivity. The GVL reactivity of 10^7 bone marrow plus 10^7 lymph node or 2×10^7 spleen cells from CBA mice was measured in a bioassay system described previously [8] and depicted in Figure 1. The bioassay was designed so that any antileukemic effect would be the exclusive result of a GVL reaction, and was used so that confounding causes of death, such as GVH disease or radiation injury, would be excluded. On day zero, 8 to 10 week old nonleukemic AKR mice (AKR 1°) were given lethal irradiation followed four hr later by i.v. injections of 10^5 viable AKR-L cells. The day following immunosuppression and leukemia inoculation the AKR primary hosts received a transplant of immunocompetent cells from unimmunized, specifically immunized, or allosensitized CBA donors. On day 7 (six days after transplant) the AKR primary hosts were killed, their spleens prepared into

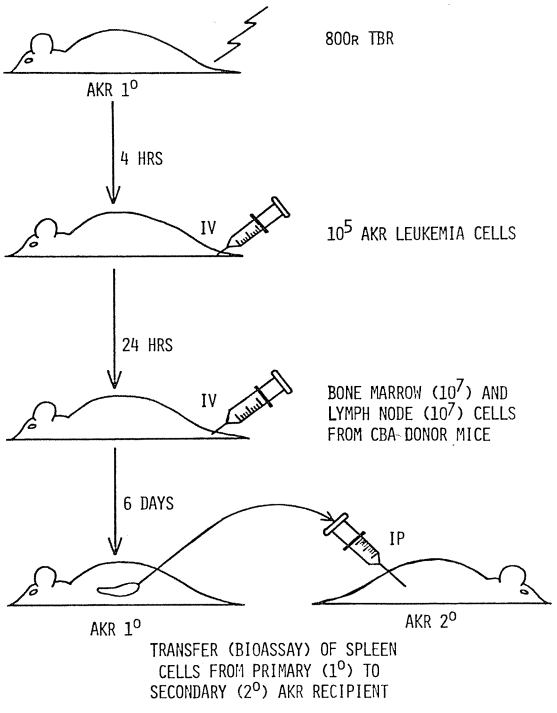


Fig. 1. Bioassay model to measure the GVL reactivity of transplanted immunocompetent cells against AKR-L cells. See text for details

single cell suspensions, and all of the spleen cells from each AKR primary host were transferred i.p. to an individual 8 to 10 week old nonleukemic AKR secondary (AKR 2°) host. The assumption was made that at least one viable leukemia cell was present in the spleen of the AKR primary hosts if the AKR secondary hosts died within 90 days. Further, if the secondary hosts survived 90 days, the assumption was made that the transplanted CBA cells had sufficient GVL reactivity to eliminate all viable leukemia cells from (at least the spleens of) the primary hosts. The theoretical and experimental bases for this bioassay and the rationale for use of the spleen as the most sensitive bioassay organ have been described [8].

Assay for GVH Reactivity. Lethally irradiated (800 r) 10 to 12 week old nonleukemic AKR mice were given i.v. injections of 10^7 bone marrow plus 10^7 lymph node or 2×10^7 spleen cells from unimmunized, specifically immunized, or allosensitized CBA donors. The AKR hosts were observed daily for clinical evidence of GVH disease and for survival. For the purposes of interpretation of the data, all deaths were attributed to GVH disease and its complications.

C. Results

The results of tests measuring the GVL and GVH reactivities of transplanted cells from unimmunized, specifically immunized, and allosensitized CBA donors are shown in Table 1. Data from control groups demonstrated that if no CBA cells were given on day 1 of the GVL bioassay (Fig. 1), viable leukemia cells were transferred from the AKR primary hosts and resulted in death of 307/308 AKR secondary recipients within 52 days (Group 1, Table 1). Administration of immunocompetent cells from unimmunized CBA donors failed to eliminate leukemia from the AKR primary hosts and all AKR secondary recipients died of

leukemia within 90 days (Group 2). Highly significant ($P<0.001$) GVL reactivity was evident when leukemic AKR primary hosts were treated with cells from specifically (AKR-L_x) immunized CBA donors (cf. Groups 3 and 2). Immunocompetent cells from each group of allosensitized CBA donors (Groups 4–8) had significantly greater GVL reactivity than did cells from unimmunized CBA (Group 2) donors ($P<0.001$). In most groups the level of GVL reactivity induced by immunization with lymphoid cells from healthy mice was comparable to that observed after specific immunization (cf. Groups 5–8 and 3). Immunization of CBA mice with lymphoid cells from either MHC compatible strains (Groups 6, 7) or MHC incompatible strains (Groups 4, 5, 8) induced GVL reactivity in the CBA donors. Allosensitization with pooled lymphoid cells obtained from seven MHC incompatible strains, as suggested by Bach et al. [1], induced a high level of GVL reactivity (Group 10).

Table 1. Survival data from assays of GVL reactivity in leukemic AKR mice and GVH reactivity in nonleukemic AKR mice[a]

		Immunogen[b]		GVL Reactivity[c]			GVH Reactivity		
Group	Donor	Source	H-2	No. of mice	MST (days)	Survival (%) at day 90	No. of mice	MST (days)	Survival (%) at day 90
1.	None	–	–	308	25	<1	155	13	2
2.	CBA	–	–	108	23	0	88	>90	63
3.	CBA	AKR-L_x	k	39	>90	92	11	11	18
4.	CBA	SJL	s	32	>90	53	24	>90	71
5.	CBA	C57BL/10	b	23	>90	74	12	>90	100
6.	CBA	B10.BR	k	40	>90	90	10	>90	50
7.	CBA	C3H/He	k	22	>90	91	8	>90	88
8.	CBA	Pool[d]	not k	12	>90	100	12	>90	83

[a] Lethally irradiated (800 r) leukemic and nonleukemic AKR (H-2^k) mice received 10^7 bone marrow plus 10^7 lymph node or 2×10^7 spleen cells from unimmunized, specifically immunized or allosensitized CBA (H-2^k) donors.

[b] CBA donor mice were immunized with six weekly i.p. injections of 10^7 lymphoid cells

[c] The bioassay shown in Figure 1 was used to measure GVL reactivity of donor cells. Survival data are for AKR secondary recipients

[d] 10^7 lymphoid cells from each of the following strains were pooled and used to immunize CBA donors: C57BL/10 (H-2^b), BALB/c (H-2^d), A.CA (H-2^f), B10.P (H-2^p), DBA/1 (H-2^q), B10.RIII (H-2^r), SJL (H-2^s)

Also shown in Table 1 are survival data from tests evaluating the GVH reactivity of immunocompetent cells from unimmunized, specifically immunized and allosensitized CBA donors in immunosuppressed, nonleukemic AKR hosts. The 63% 90-day survival rate following transplantation of 10^7 bone marrow plus 10^7 lymph node or 2×10^7 spleen cells from unimmunized CBA donors (Group 2, Table 1) was similar to data reported previously [5, 33]. Also, the finding that immunocompetent cells from specifically immunized CBA donors exhibited significantly greater GVH reactivity ($P<0.05$) than did cells from inimmunized CBA mice (cf. Groups 3 and 2) is in conformity with a previous report from our laboratory [12]. In contrast, no significant increase in GVH-related mortality

occurred when 800 r irradiated AKR hosts received transplants of immunocompetent cells from allosensitized CBA donors (cf. Groups 4–8 and 2).

D. Discussion

Two phenomena were reported here: First, GVL reactivity against AKR-L was induced in MHC compatible CBA donors by means of allosensitization; Second, allosensitization did not amplify the GVH reactivity of the donor cells. Taken together, the two phenomena may have important implications for the future application of adoptive immunotherapy as a treatment strategy for patients with acute leukemia.

On the strength of diverse observations, we [4, 9, 12] and others [15, 21, 24] have postulated the existence of at least two different subpopulations of effector cells; one capable of attacking tumor cells and another which attacks normal host cells. The data presented here which showed that GVL reactivity was independent of GVH reactivity provide strong support for this concept. In addition, the data appeared to fulfill a basic need for successful clinical application of adoptive immunotherapy: The simple and safe expedient of allosensitization of MHC compatible donors induced cells which, when transplanted, resulted in the destruction of widely disseminated leukemia cells without augmentation of their native, mild GVH reactivity.

In previous reports we described methods used to select donors for adoptive immunotherapy of experimental leukemias [12, 23]. Shown in Table 2 is another simple scoring system which can be used to rank donors. Selected data from Table 1 and from previous reports are presented to demonstrate the range of results. The »ideal« donor for adoptive immunotherapy (Group 1, Table 2) would have high GVL reactivity manifested by 100% survival of AKR bioassay secondary recipients, and absent GVH reactivity manifested by 100% survival of immunosuppressed AKR hosts; the resulting score would be 200. Reactions

Table 2. Scoring system to aid in donor selection for adoptive immunotherapy of AKR-L

Group	Donor of immunocompetent cells	GVL Reactivity[a]	GVH Reactivity[b]	Score[c]
1.	"Ideal"	100	100	200
2.	C3H/He (H-2^k)	0[d]	13[d]	13
3.	RF (H-2^k)	0	83	83
4.	SJL (H-2^s)	94[d]	0[d]	94
5.	C57BL/6 (H-2^b)	100[d]	0[d]	100
6.	CBA α AKR-L_x	92	18	110
7.	CBA α C3H/He	91	88	179
8.	CBA α H-$2^{non\text{-}k}$ Pool	100	83	183

[a] Percentage of AKR secondary hosts alive 90 days after bioassay

[b] Percentage of lethally irradiated (800 r) AKR hosts alive 90 days after transplantation

[c] Sum of GVL and GVH percentages

[d] Data from reference 12

shown in Table 2 are: low GVL and low GVH (Group 3); high GVL and high GVH (Groups 4–6); low GVL and high GVH (Group 2); and high GVL and low GVH (Groups 7, 8). The latter groups, with scores of 179 and 183, most closely approximated the ideal. The data clearly show the lack of correlation between GVL and GVH reactions and the efficacy of allosensitization to prepare MHC compatible mice as donors for adoptive immunotherapy.

E. Conclusions

1. GVL and GVH reactions appeared to be independent phenomena which were separable.
2. The data presented here suggest that allosensitization may be a clinically feasible approach to induce GVL reactivity in MHC compatible donors without intensification of GVH reactivity.
3. Allosensitization to induce GVL reactivity in MHC compatible donors circumvents any need to expose healthy donors to leukemia cells or leukemia cell products.

Acknowledgements

This work was supported by U.S. Public Health Service Grants CA-18440 and CA-20484 from the National Cancer Institute and grants from the Allen-Bradley Foundation, the Briggs and Stratton Corporation Foundation, the Leukemia Research Foundation and the Board of Trustees, Mount Sinai Medical Center. Dr. Truitt is a Scholar of the Leukemia Society of America. We thank Evangeline R. Reynolds, who supervised all of the experiments, and Eli Godfried, Lisa M. Lavery, Patricia A. Matthew, and Shirley A. Stanko for expert technical assistance.

References

1. Bach, M. L., Bach, F. H., Zarling, J. M.: Pool-priming: a means of generating T lymphocytes to tumour or virus-infected cells. Lancet 1978 *I*, 20
2. Bear, Jr., R. H., Roholt, O. A., Pressman, D.: Protection against syngeneic tumor grafts induced by inoculation with normal allogeneic tissues. Immunol. Commun. *6*, 547 (1977)
3. Boranic, M.: Transient graft versus host reaction in the treatment of leukemia in mice. J. Natl. Cancer Inst. *4*, 421 (1968)
4. Bortin, M. M.: Graft versus leukemia. In: Clinical immunobiology, Vol. II. Bach, F. H., Good, R. A. (eds.), pp. 287–306, New York: Academic Press 1974
5. Bortin, M. M., Rimm, A. A., Advisory Committee of the International Bone Marrow Transplant Registry: Bone marrow transplantation for acute myeloblastic leukemia. JAMA *240*, 1245 (1978)
6. Bortin, M. M., Rimm, A. A., Rodey, G. E., Giller, R. H., Saltzstein, E. C.: Prolonged survival in long-passage AKR leukemia using chemotherapy, radiotherapy and adoptive immunotherapy. Cancer Res. *34*, 1851 (1974)

7. Bortin, M. M., Rimm, A. A., Rose, W. C., Saltzstein, E. C.: Graft versus leukemia. V. Absence of antileukemic effect using allogeneic H-2 identical immunocompetent cells. Transplantation *18,* 280 (1974)
8. Bortin, M. M., Rimm, A. A., Saltzstein, E. C.: Graft versus leukemia. II. Quantification of adoptive immunotherapy in murine leukemia. Science *179,* 811 (1973)
9. Bortin, M. M., Rimm, A. A., Saltzstein, E. C., Rodey, G. E.: Graft versus leukemia. III. Apparent independent antihost and antileukemic activity of transplanted immunocompetent cells. Transplantation *16,* 182 (1973)
10. Bortin, M. M., Rose, W. C., Truitt, R. L., Rimm, A. A., Saltzstein, E. C., Rodey, G. E.: Graft versus leukemia. VI. Adoptive immunotherapy in combination with chemoradiotherapy for spontaneous leukemia-lymphoma in AKR mice. J. Natl. Cancer Inst. *55,* 1227 (1975)
11. Bortin, M. M., Truitt, R. L., Blasecki, J. W., Shih, C.-Y., Rimm, A. A.: Induction of reactivity against murine leukemia in MHC compatible donors. In: Graft-versus-leukemia in man and animal models. Okunewick, J. P., Meredith, R. F. (eds.). West Palm Beach: CRC Press 1979 (in press)
12. Bortin, M. M., Truitt, R. L., Rimm, A. A.: Nonspecific adoptive immunotherapy of T cell acute lymphoblastic leukemia in man. In: The handbook of cancer immunology, Vol. 5: Immunotherapy. Waters, H. (ed.), pp. 403–429. New York: Garland 1978
13. Bortin, M. M., Truitt, R. L., Rose, W. C., Rimm, A. A., Saltzstein, E. C.: Adoptive immunotherapy of spontaneous leukemia-lymphoma in AKR mice. In: Advances in experimental medicine and biology. The reticuloendothelial system in health and disease, Vol. 73B. Friedman, H., Escobar, M. R., Reichard, S. M. (eds.), pp. 331–339. New York: Plenum Press 1976
14. Chester, S. J., Esparza, A. R., Flinton, L. J., Simon, J. D., Kelley, R. J., Albala, M. M.: Further development of a successful protocol of graft-versus-leukemia without fatal graft-versus-host disease in AKR mice. Cancer Res. *37,* 3494 (1977)
15. Fernandes, G., Yunis, E. J., Good, R. A.: Depression of cytotoxic T cell subpopulations in mice by hydrocortisone treatment. Clin. Immunol. Immunopathol. 4, 304 (1975)
16. Garrido, F., Festenstein, H.: Further evidence for derepression of H-2 and Ia-like specificities of foreign haplotypes in mouse tumor cell lines. Nature *261,* 705 (1976)
17. Garrido, F., Schirrmacher, V., Festenstein, H.: H-2 like specificities of foreign haplotypes appearing on a mouse sarcoma after vaccinia virus infection. Nature *259,* 228 (1976)
18. Germain, R., Dorf, M. E., Benacerraf, B.: Inhibition of T-lymphocyte-mediated tumor-specific lysis by alloantisera directed against the H-2 serological specifities of the tumor. J. Exp. Med. *142,* 1023 (1975)
19. Invernizzi, G., Carbone, G., Meschini, A., Parmiani, G.: Multiple foreign non-H-2 determinants on the surface of a chemically-induced murine sarcoma. J. Immunogenet. *4,* 97 (1977)
20. Invernizzi, G., Parmiani, G.: Tumour-associated transplantation antigens of chemically induced sarcomata cross reacting with allogeneic histocompatibility antigens. Nature *254,* 713 (1975)
21. Kedar, E., Bonavida, B.: Studies on the induction and expression of T cell-mediated immunity. IV. Non-overlapping populations of alloimmune cytotoxic lymphocytes with specificity for tumor-associated antigens and transplantation antigens. J. Immunol. *115,* 1301 (1975)
22. The Lancet Editorial Board: Bone-marrow transplantation in leukaemia. Lancet 1977 *II,* 392
23. LeFeber, W. P., Truitt, R. L., Rose, W. C., Bortin, M. M.: Graft versus leukemia. VII. Donor selection for adoptive immunotherapy in mice. In: Experimental hematology today. Baum, S. J., Ledney, G. D. (eds.), pp. 239–246. New York: Springer 1977
24. Mage, M. G., McHugh, L. L.: Retention of graft-vs-host activity in non-adherent spleen cells after depletion of cytotoxic activity by incubation on allogeneic target cells. J. Immunol. *111,* 652 (1973)
25. Martin, W. J., Gipson, T. G., Martin, S. E., Rice, J. M.: Derepressed alloantigen on transplacentally induced lung tumor coded for by H-2 linked gene. Science *194,* 532 (1976)
26. Martin, W. J., Gipson, T. G., Rice, J. M.: H-2 associated alloantigen expressed by several transplacentally-induced lung tumours of C3Hf mice. Nature *265,* 738 (1977)
27. Mathé, G., Amiel, J. L., Schwarzenberg, L., Cattan, A., Schneider, M.: Adoptive immunotherapy of acute leukemia: Experimental and clinical results. Cancer Res. *25,* 1525 (1965)
28. Meschini, A., Invernizzi, G., Parmiani, G.: Expression of alien H-2 specificities on a chemically induced BALB/c fibrosarcoma. Int. J. Cancer *20,* 271 (1977)

29. Natori, T., Law, L. L., Appella, E.: Immunochemical evidence of a tumor-specific surface antigen obtained by detergent solubilization of the membranes of a chemically induced sarcoma, Meth-A. Cancer Res. *38,* 359 (1978)
30. Odom, L. F., August, C. S., Githens, J. H., Humbert, J. R., Morse, H., Peakman, D., Sharma, B., Rusnak, S. L., Johnson, F. B.: Remission of relapsed leukemia during a graft-versus-host reaction, a "graft-versus-leukemia reaction" in man? Lancet 1978 *II,* 537
31. Parmiani, G., Invernizzi, G.: Alien histocompatibility determinants on the cell surface of sarcomas induced by methylcholanthrene. I. In vivo studies. Int. J. Cancer *16,* 750 (1975)
32. Prat, M., Rogers, M. J., Appella, E.: Alloantisera reacting with tumor cells of inappropriate haplotype. I. Characterization of target antigens. J. Natl. Cancer Inst. *61,* 527 (1978)
33. Rodey, G. E., Bortin, M. M., Bach, F. H., Rimm, A. A.: Mixed leukocyte culture reactivity and chronic graft versus host reactions (secondary disease) between allogeneic H-2^k mouse strains. Transplantation *17,* 84 (1974)
34. Santos, G. W.: Bone marrow transplantation in acute leukemia – remaining problems. Transplant. Proc. 10, 173 (1978)
35. Thomas, E. D., Buckner, C. D., Banaji, M., et al.: One hundred patients with acute leukemia treated by chemotherapy, total body irradiation and allogeneic marrow transplantation. Blood *49,* 511 (1977)
36. Truitt, R. L.: Application of germfree techniques to the treatment of leukemia in AKR mice by allogeneic bone marrow transplantation. In: The handbook of cancer immunology, Vol. 5: Immunotherapy. Waters, H. (ed.), pp. 431–452. New York: Garland 1978
37. Truitt, R. L.: Use of decontamination and a protected environment to prevent secondary disease following adoptive immunotherapy of acute leukemia in mice. In: Experimental hematology today, 2nd ed. Baum, S. J., Ledney, G. D. (eds.), pp. 195–201. New York: Springer 1978
38. Truitt, R. L., Bortin, M. M., Rimm, A. A.: Successful adoptive immunotherapy of AKR spontaneous T cell leukemia following antibiotic decontamination and protective isolation. In: Clinical and experimental gnotobiotics. Fliedner, T. M., Heit, H., Niethammer, D., Pflieger, H. (eds.), pp. 215–220. Stuttgart: Fischer 1979
39. Truitt, R. L., Rimm, A. A., Saltzstein, E. C., Rose, W. C., Bortin, M. M.: Graft-versus-leukemia for AKR spontaneous leukemia-lymphoma. Transplant. Proc. *8,* 569 (1976)
40. UCLA Bone-Marrow Transplantation Team: Bone-marrow transplantation in acute leukaemia. Lancet 1977 *II,* 1197
41. Wrathmell, A. B., Gauci, C. L., Alexander, P.: Cross reactivity of an alloantigen present on normal cells with the tumour-specific transplantation-type antigen of the acute myeloid leukaemia (SAL) of rats. Br. J. Cancer *33,* 187 (1976)
42. Zarling, J. M., Robins, H. I., Raich, P. C., Bach, F. H., Bach, M. L.: Generation of cytotoxic T lymphocytes to autologous human leukaemia cells by sensitization to pooled allogeneic normal cells. Nature *274,* 269 (1978)

Discussion

Wagner: Allo-immunization across H-2 resulted in CTL reactivity against H-2 compatible leukemias. Does this mean that alloreactive killer cells contain H-2 restricted killer cells? In other words, if you immunize against a pool of alloantigens you certainly will have some clones which react against H-2 in an H-2 restricted fashion.

Bortin: We do not know that donor cytotoxic T lymphocytes were responsible for the killing. We do know that anti-theta plus complement treatment of donor cells abolished killing (Bortin, et al. Nature *281,* 490, 1979), but it is uncertain whether the type of donor cell inactivated by this treatment was a cytotoxic, helper or suppressor T cell or perhaps an NK cell. Experiments addressing this question are in progress. We do not know whether H-2 restriction at the target cell level was operative in this system.

Mathé: The mechanism which induced GVH is mediated by T cell reactivity, while for GVH you have a combination of cells, T, B, K and so on, therefore can't you imagine that by getting your reaction with GVH you may induce suppressor cells which work mainly on the T machinery and less on the combined machinery for GVH.

Bortin: Yes. But it is important to keep in mind that cells from unprimed CBA donors had no GVL reactivity and after allosensitization they had high GVL reactivity, but there was no change in GVH reactivity. One explanation is that the GVL and GVH reactions were carried out by two different subsets of effector cells. It is conceivable, as you proposed, that cells which suppressed GVH reactivity and stimulated GVL reactivity were generated as a consequence of allosensitization.

2 Experimental Bone Marrow Transplantation in Dogs

Marrow Graft Studies in Dogs*

R. Storb, P. L. Weiden, H. J. Deeg**, B. Torok-Storb**, K. Atkinson, T. C. Graham, and E. D. Thomas**

This report summarizes some of our preclinical studies of marrow grafting in the dog:

A. Immunosuppressive Conditioning Regimens

Most commonly we have used total body irradiation (TBI) delivered from 2 opposing ^{60}Co sources at 9.3 R/minute. Consistent marrow engraftment in dogs occurred only at or above 1200 R midline air (900–1,000 rads midline tissue) exposure [27, 41]. Dogs grafted after 1200 R TBI have always been complete chimeras, that is all metaphases analyzed in marrow, lymph node and peripheral blood showed the donor karyotype [4, 12, 27]. Red blood cell antigen and enzyme phenotypes as well as pulmonary macrophages proved to be of donor origin [27, 48, 50]. This feature of consistent and complete chimerism has made TBI a major component of the conditioning regimens for marrow grafting of patients with hematologic malignancies [44]. More recently we have begun increasing the TBI exposure by the use of fractionated irradiation. The dose per fraction was 200 R, the fractionation intervals were at least 3 hours. With this approach, total doses up to 2400–2800 R have been tolerated by dogs without fatal gastrointestinal toxicity. These studies have served as the basis for a similar approach in patients with leukemia in relapse.

In agreement with studies in rodents and monkeys [14, 21] the alkylating agent cyclophosphamide (CY) can be substituted for TBI to condition dogs for marrow grafting [20]. In contrast to radiation chimeras, CY chimeras showed persisting mixtures of host and donor hemopoietic cells. The persistence of host cells in CY chimeras may be undesirable in efforts to treat hemopoietic malignancies by marrow grafting but can be disregarded when treating non-malignant disorders of the marrow such as aplastic anemia [32, 43].

Extensive toxicologic, immunologic and marrow transplantation studies in dogs were carried out with the alkylating agent dimethyl myleran (DMM) [10]. Although the agent produced profound marrow depression, marrow grafts could

---

* This investigation was supported by grant Nos. CA 18047, CA 15704, CA 09319 from the National Cancer Institute, DHEW

** Dr. Deeg is a Fellow of the Leukemia Society of America and is supported in part by a University of Washington Graduate School Research Project. Dr. Torok-Storb is supported by a Research Science Award, AM-05356. Dr. Thomas is a recipient of a Research Career Award AI 02425 from the National Institutes of Allergy and Infectious Diseases

not be achieved across a major histocompatibility barrier except in some dogs given antithymocyte serum (ATS) in addition to the DMM. Subsequent studies in DLA-identical littermates have shown that a dose of 10 mg of DMM/kg was sufficiently immunosuppressive to permit sustained engraftment in 50% of the dogs studied [36]. 50% of the dogs rejected the graft, presumably as a result of resistance to "minor" histocompatibility systems not associated with DLA. The addition of ATS or procarbazine and ATS to DMM successfully abrogated resistance. Its profound antitumor activity with marrow toxicity reversible by marrow transplantation combined with its moderate immunosuppressive properties suggest that DMM is useful in combination with other agents to condition human patients with hematologic malignancy for marrow transplantation.

B. Histocompatibility Typing

The dog was the first random bred animal in which the predictive value of in vitro histocompatibility testing for the outcome of marrow grafts was demonstrated [5, 18, 25, 30]. Compatibility appears to be an important factor governing graft rejection, development of lethal graft-versus-host disease (GVHD) or eventual survival of the recipient after the marrow graft. Littermates identical for the serotypically defined determinants DLA-A and B and mutually non-reactive in mixed leukocyte culture (MLC) survived better than mismatched ones but approximately 50% of them succumbed to late GVHD [25, 30]. This indicated that "minor" histocompatibility differences also can play a part in the development of fatal GVHD and emphasized the need for immunosuppression post-grafting even in this "compatible" situation. Based on the dog studies we have carried out human marrow grafts in patients with family members identical for the genetic determinants of the MHC. Further studies in unrelated dogs of different breed, however, suggested that successful long-term survival can be achieved in some recipients of histocompatible marrow [37]. Details of the studies in unrelated dogs are described below.

C. Resistance to Marrow Grafts in Dogs Mediated by Antigens Close to but not Identical with DLA-A, -B and -D

We have previously shown that grafts with low numbers of marrow cells (less than 4×10^8 cells/kg) after 1200 R TBI are successful in DLA identical littermates (20 of 21 dogs engrafted) but not in DLA nonidentical dogs [38, 55] (none of 8 littermates and 1 of 16 unrelated dogs engrafted) suggesting a locus of resistance associated with the major histocompatibility complex. Attempts to abrogate resistance by the macrophage inhibitor silica or by added immunosuppression with ATS, procarbazine or MTX all failed [38, 55]. Only when viable donor peripheral blood leukocytes were added to the marrow inoculum was resistance consistently overcome (79 of 83 littermates and 69 of 75 unrelated dogs engrafted), perhaps because of the administration of increased numbers of stem cells [17–19] from the peripheral blood or, more likely, an effect of donor

lymphocytes. When the leukocytes were irradiated in vitro before infusion, the effect was abrogated [3].

Subsequent studies were designed to determine (1) whether the antigens mediating resistance are coded for by DLA-A, -B or -D and (2) whether the addition of thoracic duct lymphocytes known not to contain hemopoietic stem cells [3] to the low dose marrow inoculum would overcome resistance. Results and conclusions were as follows:

1. Fifteen dogs were given $\leq 4 \times 10^8$ marrow cells/kg from unrelated donors that were DLA-A, -B and -D homozygous and identical with the recipient. In contrast to results in littermates, 7 of the 15 rejected their graft and only 8 had sustained engraftment suggesting that the genetic determinants for resistance are separate from but in linkage disequilibrium with DLA-A, -B and -D.
2. Eight dogs received thoracic duct lymphocytes in addition to the marrow inoculum from unrelated DLA non-identical donors and all showed sustained hemopoietic engraftment. Clearly, viable donor lymphocytes were able to abrogate allogeneic resistance by either actively suppressing residual host immunity or, alternatively, increasing hemopoiesis by cell-cell interaction with hemopoietic cells in the infused marrow. The latter mechanism is suggested by studies in vitro indicating that thoracic duct lymphocytes from dogs co-cultured with autologous marrow significantly increased the number of erythroid colonies over that obtained with marrow alone [3, 45]. Identification and isolation of the thoracic duct cells mediating the enhancing effect on hemopoiesis may make possible successful marrow grafts across major canine histocompatibility barriers without an increased risk of graft-vs-host disease.

D. Sensitization to Marrow Grafts by Blood Transfusions

Previous studies in dogs given 1200 R and a hemopoietic graft from DLA identical littermates have shown that marrow graft rejection generally does not occur in untransfused recipients (58 of 59 achieved sustained engraftment) but is seen after a single transfusion of whole blood from the marrow donor on day −10 (13 of 18 rejected) and even after multiple transfusions of random whole blood (3 of 11 rejected) [22, 26]. We extended those initial observations by determining the incidence of rejection of DLA-identical littermate marrow grafts following administration of different cells from the marrow donor on days −24, −17 and −10 before transplantation [40]. With this protocol the following results and conclusions were established: 1) All 19 dogs given transfusions of whole blood rejected their grafts. This 100% incidence of rejection after 3 transfusions indicates that more than 1 minor histocompatibility system outside of DLA is involved in transfusion induced sensitization and subsequent marrow graft rejection (the probability of encountering 19 of 19 rejections if only 1 polymorphic locus were involved is <0.008 [binomial significance test]); 2) 6 dogs given subcutaneous injections of cultured skin epithelial cells rejected their subsequent marrow grafts. Hence, antigens mediating rejection are not restricted to hemopoietic cells but are also expressed on at least one other tissue; 3) 7 of 15 dogs given transfusions of platelet concentrates (white and red blood cell poor)

rejected the marrow graft while 8 showed sustained engraftment; 4) 5 of 14 dogs given transfusions of red blood cells (white blood cells and platelet poor) rejected the graft while 9 had sustained engraftment. This suggests that only some (and perhaps none) of the non-DLA antigens responsible for rejection reside on platelets and red blood cells. To definitively answer this question, the current blood cell separation techniques must be improved further to provide pure red blood cells and platelets for transfusion studies; 5) Preliminary data suggest that the antigens involved in rejection do not reside on granulocytes but are expressed on a mononuclear cell population in the peripheral blood.

Assays of lymphocytotoxic antibodies, mixed leukocyte culture reactivity and survival of donor platelets in the recipient did not predict the fate of the subsequent marrow graft. Among the techniques used, only the results of lymphocyte marrow co-cultures [46] proved to be predictive of the fate of a subsequent marrow graft. Twenty pairs of transfused DLA identical littermates in the present study were examined. Fourteen of the 20 rejected the marrow graft. In 13 of the 14 rejection was predicted by the in vitro observation that recipient lymphocytes either failed to stimulate or inhibited erythroid colony formation by donor marrow. Successful and sustained marrow engraftment occurred in 6 dogs. In 5 of these this was associated with significant stimulation of donor marrow in co-culture. This correlation was highly significant and suggests that transfusion induced sensitization and marrow graft rejection can be predicted very accurately by reduced erythroid colony growth of donor marrow co-cultured with recipient lymphocytes. In view of the many blood and platelet transfusions used to treat patients with aplastic anemia it is quite likely that prior sensitization plays a detrimental role in human marrow grafting [32, 39, 43]. The current findings in DLA identical canine littermate marrow recipients are of potential practical importance for the planning of platelet and red blood cell support of human marrow graft candidates.

E. GVHD

GVHD is quite similar in random bred species; dogs, monkeys and man [7, 13, 27, 30, 58]. The clinical and histologic changes are a result of disturbances in solid organs, in particular the skin, gut, liver and the lymphoid tissues. GVHD is rapidly fatal in dogs given marrow grafts from histoincompatible or random unrelated donors and not treated with immunosuppression following grafting. GVHD can be prevented or reduced in severity by the prophylactic use of MTX [23, 24, 30, 42]. The drug is given intermittently after grafting and has been discontinued in some dogs after approximately 3 months without subsequent development of fatal GVHD. 6-mercaptopurine was effective but inferior to MTX in the dog. Procarbazine, Cy, cytosine arabinoside and prednisone were ineffective. ATS prepared in rabbits had only marginal beneficial effects on GVHD when given to recipients before grafting [9] and no beneficial effect when given immediately after grafting. ATS was found to be of value, however, in treating established GVHD [31]. MTX (prophylactically) and ATS (for established GVHD) are currently used in our human marrow transplant program [33, 56] based on the studies in dogs.

F. GVHD, a Phenomenon Linked to But not Exclusively Determined by Known Antigens of the Canine Major Histocompatibility Complex

Marrow grafts between DLA identical canine littermates have, as a rule, resulted in successful engraftment [30]. GVHD owing to "minor" histocompatibility differences outside of DLA could be successfully overcome by administration of intermittent MTX for 100 days post grafting [30]. GVHD has, however, been a formidable problem when grafts between DLA non-identical donor recipient combinations were carried out [28]. This has led to the suggestion that DLA antigens detected by serologic histocompatibility typing and MLC are the determinants influencing the outcome of a marrow transplant. More recent data from our laboratory have provided suggestive evidence that 1 or more loci of the MHC in addition to DLA-A, -B and -D are involved in GVHD [34, 37].

These studies involved dogs given 1200 R TBI, a hemopoietic graft consisting of a combination of marrow and donor leukocytes, and post grafting MTX for 102 days. Of 17 DLA identical littermates treated in this fashion, none developed GVHD, and 16 survived. In contrast, 43 of 54 DLA non-identical littermates died of complications associated with GVHD, and only 2 survived. Eight of 11 DLA heterozygous dogs given grafts from DLA homozygous littermates died with GVHD and only 1 survived. Surprisingly, 6 of 14 DLA homozygous dogs given grafts from DLA heterozygous littermates died with GVHD and only 2 survived. Six of 13 dogs given grafts from homozygous and DLA identical unrelated dogs died with GVHD and 4 survived.

These results are incompatible with the concept that solely the loci detected by MLC and serotyping are responsible for GVHD. One or more additional loci appear to be involved. Knowledge of this locus (loci) is important if marrow grafting between unrelated individuals is to be successful. However, results also indicate that an unrelated "compatible" marrow graft is more likely to succeed than a graft from an incompatible littermate.

G. Immunologic Studies After Grafting

Extensive studies of humoral and cell-mediated immunity have been carried out in canine radiation chimeras [12]. Dogs were found to be profoundly immunodeficient for 200–300 days after marrow grafting. Thereafter immunologic reconstitution appeared to be complete. As a clinical correlate to these studies on immune function, long-term chimeras regained their health and are able to live in an unprotected environment without increased susceptibility to infection. The findings of ultimate immunologic recovery was encouraging in regards to human marrow grafting.

H. Graft-Host Tolerance

Many healthy long-term marrow chimeras have been obtained with chimerism persisting for now up to 10 years. These animals have been used in studies on the nature of the operational tolerance necessary to maintain the stable chimeric state

[35, 47]. The studies have shown that serum blocking factors are not involved in maintaining the stable chimeric state in canine radiation chimeras. Lymphocytes from long-term DLA-nonidentical chimeras failed to respond to stored host lymphocytes in MLC but responded to lymphocytes from unrelated dogs and to phytohemagglutinin. In contrast, marrow donor lymphocytes responded well to host lymphocytes. The specific non-responsiveness of this and other mismatched chimeras could also not be attributed to serum blocking factors, since the MLC tests were carried out with pooled normal dog serum. Furthermore, cell mixing experiments using various concentrations of chimera and donor lymphocytes as responding cells in MLC failed to show the presence of circulating suppressor cells (K. Atkinson et al., unpublished observations). These data indicate that stable chimeras lack circulating mononuclear cells responsive to host antigens in MLC. These results are in apparent contrast to an in vivo study involving infusion of marrow donor lymphocytes into stable radiation chimeras [52]. In this study, infusion of lymphocytes from non-sensitized marrow donors failed to produce GVHD. Only lymphocytes from donors specifically sensitized against the chimeras by chimeric skin grafts resulted in GVHD in 75% of the dogs. We interpreted these results to suggest the presence of an active mechanism in the chimeras suppressing recognition of host antigens by infused donor lymphocytes and development of GVHD. The mechanism apparently could be overcome by the infusion of sensitized donor cells. Our in vitro results, however, suggest that the cells mediating this suppression do not circulate in the peripheral blood.

I. Neoplasias and Late Radiation Effects in Long-term Chimeras

More than 100 chimeras have been observed for prolonged periods of time [27] with some of them now surviving for up to 10 years. A comparison of the cumulative cancer incidence between 108 long-term radiation chimeras and 215 unirradiated control dogs did show a difference in the overall incidence of cancer (H. J. Deeg et al., Blood, in press). Also, cancer tended to occur significantly earlier in the radiation chimeras, on the average 3–5 years. The tumors seen in chimeras were not different from those seen in the control population and involved breast carcinoma, ovarian carcinoma, hypernephroma, perianal gland carcinoma, etc. We have not observed a preponderance of malignancies of the reticuloendothelial system as has been described in some rodent strains [1, 16]. These data in dogs suggest that the occurrence of malignant tumors is accelerated in radiation chimeras. TBI, therefore, in man should be restricted for the conditioning of patients with malignant diseases while patients with otherwise non malignant disease such as aplastic anemia should receive conditioning regimens consisting of chemotherapeutic agents. No case of cancer has been seen in 16 canine CY or DMM chimeras observed for prolonged periods of time.

A problem arising within the first year of irradiation has been pancreatic fibrosis accompanied by maldigestion. This has also been seen in dogs with autologous marrow grafts. We have not carried out careful studies of gonadal function in chimeras. As a rule, chimeras appear to be sterile, yet occasional TBI

chimeras have become impregnated while others have sired normal litters [8]. CY chimeras appear to have normal fertility. No radiation effects on other organs have so far been identified. Obviously more long-term survivors need to be kept for evaluating late radiation effects and the development of tumors.

J. Marrow Grafting for the Treatment of Canine Diseases

Diseases such as cyclic neutropenia, hemophilia and hemolytic anemia associated with congenital pyruvate kinase deficiency as well as spontaneous malignancies including lymphoma, leukemia and non-hematopoietic solid tumors are valuable models to study the use of marrow grafting for the treatment of these diseases. For instance, it was possible to show that canine cyclic neutropenia is not due to a defect of marrow regulation, but rather to a stem cell defect that can be corrected by marrow transplantation [2, 49]. In canine hemophilia, orthotopic transplantation of a normal liver into a hemophilic recipient completely corrected the factor VIII deficiency [11]. However there are important extrahepatic sources of factor VIII, since a hepatectomized normal dog bearing a transplanted liver from a hemophilic dog showed factor VIII levels equivalent to that seen in the heterozygous state. Marrow grafting studies ruled out the hemopoietic and lymphoid systems as sources of factor VIII production [29]. Canine lymphosarcoma and solid tumors have been treated by autologous and allogeneic marrow transplantation following TBI [6, 51, 54, 57]. Severe life-threatening hemolytic anemia due to pyruvate kinase deficiency has been corrected by marrow transplantation from DLA-identical littermates [53]. These studies have shown that hemosiderosis and myeloid hyperplasia are phenomena secondary to the hemolytic anemia rather than separate genetic defects (P. Weiden et al., unpublished observations).

References

1. Cornelius, E. A.: Rapid viral induction of murine lymphomas in the graft-versus-host reaction. J. Exp. Med. *136,* 1533–1544 (1972)
2. Dale, D. C., Graw, R. G., Jr.: Transplantation of allogeneic bone marrow in canine cyclic neutropenia. Science *183,* 83–84 (1974)
3. Deeg, H. J., Storb, R., Weiden, P. L., Shulman, H. M., Graham, T. C., Torok-Storb, B. J., Thomas, E. D.: Abrogation of resistance to and enhancement of DLA-nonidentical unrelated marrow grafts in lethally irradiated dogs by thoracic duct lymphocytes. Blood *53,* 552–557 (1979)
4. Epstein, R. B., Bryant, J., Thomas, E. D.: Cytogenetic demonstration of permanent tolerance in adult outbred dogs. Transplantation *5,* 267–272 (1967)
5. Epstein, R. B., Storb, R., Ragde, H., Thomas, E. D.: Cytotoxic typing antisera for marrow grafting in littermate dogs. Transplantation *6,* 45–58 (1968)
6. Epstein, R. B., Graham, T. C., Storb, R., Thomas, E. D.: Studies of marrow transplantation, chemotherapy and cross-circulation in canine lymphosarcoma. Blood *37,* 349–359 (1971)
7. Glucksberg, H., Storb, R., Fefer, A., Buckner, C. D., Neiman, P. E., Clift, R. A., Lerner, K. G., Thomas, E. D.: Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. Transplantation *18,* 295–304 (1974)
8. Hager, E. B., Thomas, E. D., Ferrebee, J. W.: Fertility of dogs after recovery from "lethal" exposure to radiation. Radiobiol. Radiother. (Berl.) *3,* 1–3 (1962)

9. Kolb, H. J., Storb, R., Graham, T. C., Kolb, H., Thomas, E. D.: Antithymocyte serum and methotrexate for control of graft-versus-host disease in dogs. Transplantation *16,* 17–23 (1973)
10. Kolb, H. J., Storb, R., Weiden, P. L., Ochs, H. D., Kolb, H., Graham, T. C., Floersheim, G. L., Thomas, E. D.: Immunologic, toxicologic and marrow transplantation studies in dogs given dimethyl myleran. Biomedicine *20,* 341–351 (1974)
11. Marchioro, T. L., Hougie, C., Ragde, H., Epstein, R. B., Thomas, E. D.: Organ homografts for hemophilia. Transplant. Proc. *1,* 316–320 (1969)
12. Ochs, H. D., Storb, R., Thomas, E. D., Kolb, H. J., Graham, T. C., Mickelson, E., Parr, M., Rudolph, R. H.: Immunologic reactivity in canine marrow graft recipients. J. Immunol. *113,* 1039–1057 (1974)
13. Sale, G. E., Storb, R., Kolb, H.: Histopathology of hepatic acute graft-versus-host disease in the dog. A double blind study confirms the specificity of small bile duct lesions. Transplantation *26,* 103–106 (1978)
14. Santos, G. W., Owens, A. H., Jr.: Allogeneic marrow transplants in cyclophosphamide treated mice. Transplant. Proc. *1,* 44–46 (1969)
15. Schroeder, M. L., Storb, R., Graham, T. C., Weiden, P. L.: Canine radiation chimeras: An attempt to demonstrate serum blocking factors by an in vivo approach. J. Immunol. *114,* 540–541 (1975)
16. Schwartz, R. S., Beldotti, L.: Malignant lymphomas following allogeneic disease: Transition from an immunological to a neoplastic disorder. Science *149,* 1511–1514 (1965)
17. Storb, R., Epstein, R. B., Ragde, H., Bryant, J., Thomas, E. D.: Marrow engraftment by allogeneic leukocytes in lethally irradiated dogs. Blood *30,* 805–811 (1967)
18. Storb, R., Epstein, R. B., Bryant, J., Ragde, H., Thomas, E. D.: Marrow grafts by combined marrow and leukocyte infusions in unrelated dogs selected by histocompatibility typing. Transplantation *6,* 587–593 (1968)
19. Storb, R., Epstein, R. B., Thomas, E. D.: Marrow repopulating ability of peripheral blood cells compared to thoracic duct cells. Blood *32,* 662–667 (1968)
20. Storb, R., Epstein, R. B., Rudolph, R. H., Thomas, E. D.: Allogeneic canine bone marrow transplantation following cyclophosphamide. Transplantation *7,* 378–386 (1969)
21. Storb, R., Buckner, C. D., Dillingham, L. A., Thomas, E. D.: Cyclophosphamide regimens in rhesus monkeys with and without marrow infusion. Cancer Res. *30,* 2195–2203 (1970)
22. Storb, R., Epstein, R. B., Rudolph, R. H., Thomas, E. D.: The effect of prior transfusion on marrow grafts between histocompatible canine siblings. J. Immunol. *105,* 627–633 (1970)
23. Storb, R., Epstein, R. B., Graham, T. C., Thomas, E. D.: Methotrexate regimens for control of graft-versus-host disease in dogs with allogeneic marrow grafts. Transplantation *9,* 240–246 (1970)
24. Storb, R., Graham, T. C., Shiurba, R., Thomas, E. D.: Treatment of canine graft-versus-host disease with methotrexate and cyclophosphamide following bone marrow transplantation from histoincompatible donors. Transplantation *10,* 165–172 (1970)
25. Storb, R., Rudolph, R. H., Thomas, E. D.: Marrow grafts between canine siblings matched by serotyping and mixed leukocyte culture. J. Clin. Invest. *50,* 1272–1275 (1971)
26. Storb, R., Rudolph, R. H., Graham, T. C., Thomas, E. D.: The influence of transfusions from unrelated donors upon marrow grafts between histocompatible canine siblings. J. Immunol. *107,* 409–413 (1971)
27. Storb, R., Thomas, E. D.: Bone marrow transplantation in randomly bred animal species and in man. In: Proceedings of the Sixth Leucocyte Culture Conference. Schwarz, M. R. (ed.), pp. 805–840. New York: Academic Press 1972
28. Storb, R., Kolb, H. J., Graham, T. C., LeBlond, R., Kolb, H., Lerner, K. G., Thomas, E. D.: Marrow grafts between histoincompatible canine family members. Europ. J. Clin. Biol. Res. *17,* 680–685 (1972)
29. Storb, R., Marchioro, T. L., Graham, T. C., Willemin, M., Hougie, C., Thomas, E. D.: Canine hemophilia and hemopoietic grafting. Blood *40,* 234–238 (1972)
30. Storb, R., Rudolph, R. H., Kolb, H. J., Graham, T. C., Mickelson, E., Erickson, V., Lerner, K. G., Kolb, H., Thomas, E. D.: Marrow grafts between DL-A-matched canine littermates. Transplantation *15,* 92–100 (1973)
31. Storb, R., Kolb, H. J., Graham, T. C., Kolb, H., Weiden, P. L., Thomas, E. D.: Treatment of

established graft-versus-host disease in dogs by antithymocyte serum or prednisone. Blood *42,* 601–609 (1973)

32. Storb, R., Thomas, E. D., Buckner, C. D., Clift, R. A., Johnson, R. L., Fefer, A., Glucksberg, H., Giblett, E. R., Lerner, K. G., Neiman, P.: Allogeneic marrow grafting for treatment of aplastic anemia. Blood *43,* 157–180 (1974)
33. Storb, R., Gluckman, E., Thomas, E. D., Buckner, C. D., Clift, R. A., Fefer, A., Glucksberg, H., Graham, T. C., Johnson, F. L., Lerner, K. G., Neiman, P. E., Ochs, H.: Treatment of established human graft-versus-host disease by antithymocyte globulin. Blood *44,* 57–75 (1974)
34. Storb, R., Weiden, P. L., Schroeder, M. L., Graham, T. C., Lerner, K. G., Thomas, E. D.: Marrow grafts between canine littermates homozygous or heterozygous for lymphocyte-defined histocompatibility antigens. Transplantation *21,* 299–306 (1976)
35. Storb, R., Tsoi, M. S., Weiden, P. L., Graham, T. C., Thomas, E. D.: Studies on the mechanism of stable graft-host tolerance in canine and human radiation chimeras. Transplant. Proc. *8,* 561–564 (1976)
36. Storb, R., Weiden, P. L., Graham, T. C., Lerner, K. G., Nelson, N., Thomas, E. D.: Hemopoietic grafts between DLA identical canine littermates following dimethyl myleran. Evidence for resistance to grafts not associated with DLA and abrogated by antithymocyte serum. Transplantation *24,* 349–357 (1977)
37. Storb, R., Weiden, P. L., Graham, T. C., Lerner, K. G., Thomas, E. D.: Marrow grafts between DLA-identical and homozygous unrelated dogs. Evidence for an additional locus involved in graft-versus-host disease. Transplantation *24,* 165–174 (1977)
38. Storb, R., Weiden, P. L., Graham, T. C., Thomas, E. D.: Failure of engraftment and graft-versus-host disease after canine marrow transplantation. Two phenomena linked to but not exclusively determined by known antigens of the major histocompatibility complex. Transplant. Proc. *10,* 113–118 (1978)
39. Storb, R., Thomas, E. D. for the Seattle Marrow Transplant Team: Marrow transplantation for treatment of aplastic anemia. In: Clinics in Haematology. Thomas, E. D. (ed.), pp. 597–609. London: Saunders 1978
40. Storb, R., Deeg, H. J., Weiden, P. L., Graham, T. C., Atkinson, K. A., Slichter, S. J., Thomas, E. D.: Marrow graft rejection in DLA identical canine littermates: Antigens involved are expressed on leukocytes and skin epithelial cells but probably not on platelets and red blood cells. Transplant. Proc. *11,* 504–506 (1979)
41. Thomas, E. D., Ashley, C. A., Lochte, H. L., Jr., Jaretzki, A., III, Sahler, O. D., Ferrebee, J. W.: Homografts of bone marrow in dogs after lethal total-body irradiation. Blood *14,* 720–736 (1959)
42. Thomas, E. D., Collins, J. A., Herman, E. C., Jr., Ferrebee, J. W.: Marrow transplants in lethally irradiated dogs given methotrexate. Blood *19,* 217–228 (1962)
43. Thomas, E. D., Buckner, C. D., Storb, R., Neiman, P. E., Fefer, A., Clift, R. A., Slichter, S. J., Funk, D. D., Bryant, J. I., Lerner, K. G.: Aplastic anaemia treated by marrow transplantation. Lancet *1972 I,* 284–289
44. Thomas, E. D., Storb, R., Clift, R. A., Fefer, A., Johnson, F. L., Neiman, P. E., Lerner, K. G., Glucksberg, H., Buckner, C. D.: Bone-marrow transplantation. N. Engl. J. Med. *292,* 832–843, 895–902 (1975)
45. Torok-Storb, B. J., Storb, R., Graham, T. C., Prentice, R. L., Weiden, P. L., Adamson, J. W.: Erythropoiesis in vitro: Effect of normal versus "transfusion-sensitized" mononuclear cells. Blood *52,* 706–711 (1978)
46. Torok-Storb, B. J., Storb, R., Deeg, H. J., Graham, T. C., Wise, C., Weiden, P. L., Adamson, J. W.: Growth in vitro of donor marrow cultures with recipient lymphocytes predicts the fate of marrow grafts in transfused DLA-identical dogs. Blood *53,* 104–108 (1979)
47. Tsoi, M. S., Storb, R., Weiden, P. L., Graham, T. C., Schroeder, M. L., Thomas, E. D.: Canine marrow transplantation: Are serum blocking factors necessary to maintain the stable chimeric state? J. Immunol. *114,* 531–539 (1975)
48. Weiden, P., Storb, R., Kolb, H. J., Graham, T., Anderson, J., Giblett, E.: Genetic variation of red blood cell enzymes in the dog: Use of soluble glutamic oxaloacetic transaminase as proof of chimerism. Transplantation *17,* 115–120 (1974)
49. Weiden, P., Robinett, B., Graham, T. C., Adamson, J. W., Storb, R.: Canine cyclic neutropenia. A stem cell defect. J. Clin. Invest. *53,* 950–953 (1974)

50. Weiden, P. L., Storb, R., Tsoi, M. S.: Marrow origin of canine alveolar macrophages. J. Reticuloendothel. Soc. *17,* 342–345 (1975)
51. Weiden, P. L., Storb, R., Lerner, K. G., Kao, G. F., Graham, T. C., Thomas, E. D.: Treatment of canine malignancies by 1200 R total body irradiation and autologous marrow grafts. Exp. Hematol. *3,* 124–134 (1975)
52. Weiden, P. L., Storb, R., Tsoi, M. S., Graham, T. C., Lerner, K. G., Thomas, E. D.: Infusion of donor lymphocytes into stable canine radiation chimeras: Implications for mechanism of transplantation tolerance. J. Immunol. *116,* 1212–1219 (1976)
53. Weiden, P. L., Storb, R., Graham, T. C., Schroeder, M. L.: Severe hereditary haemolytic anaemia in dogs treated by marrow transplantation. Br. J. Haematol. *33,* 357–362 (1976)
54. Weiden, P. L., Storb, R., Shulman, H., Graham, T. C.: Dimethyl myleran and autologous marrow grafting for the treatment of spontaneous canine lymphoma. Eur. J. Cancer *13,* 1411–1415 (1977)
55. Weiden, P. L., Storb, R., Graham, T. C., Sale, G. E., Thomas, E. D.: Resistance to DLA-nonidentical marrow grafts in lethally irradiated dogs. Transplant. Proc. *9,* 285–288 (1977)
56. Weiden, P. L., Doney, K., Storb, R., Thomas, E. D.: Anti-human thymocyte globulin (ATG) for prophylaxis and treatment of graft-versus-host disease in recipients of allogeneic marrow grafts. Transplant. Proc. *10,* 213–216 (1978)
57. Weiden, P. L., Storb, R., Sale, G. E., Graham, T. C., Thomas, E. D.: Allogeneic hematopoietic grafts after total body irradiation in dogs with spontaneous tumors. J. Natl. Cancer. Inst. *61,* 353–357 (1978)
58. Van Bekkum, D. W., de Vries, M. J.: Radiation Chimaeras. Radiobiological Institute of the Organisation for Health Research TNO. Rijswijk Z. H., Netherlands. New York: Academic Press 1967

Discussion

Kersey: What about the cancer incidence of cytoxan-induced chimeras?
Storb: We only have 16 animals observed for long periods of time, cytoxan and dimethylmyleran chimeras, none of those has developed cancer.
Slavin: Before you infused your chimeras with donor-type cells, did you irradiate the chimeras?

If you did not, it is difficult to exclude absence of engraftment which would explain why you did not see GVH.
Storb: We did not irradiate those animals before infusion. This is a criticism you can make, although I would not expect that syngeneic cells are being rejected. We did see prompt GVH when we gave the donor a skin graft from the chimera which the donor rejected. In other words, the sensitized cells were perfectly able to establish themselves and cause fatal GVH. We have, however, some evidence using splenic transplants that there is a transplantable cell in the spleen that, if you wish, confers tolerance. This would be compatible with the idea of a splenic suppressor cell.
Santos: Are you aware of the work of Nakić back in the fifties and most of the old rodent doctors know it, where it was shown quite clearly that if you give extra lymphoid cells you had more GVH, even though clinically this in itself was immunosuppressive. Rather than getting romantic about cooperation between lymphoid cells I would say this is an alternative explanation.
Storb: To cite other rodent literature there is also ample evidence in the syngeneic situation that the addition of thymocytes would enhance engraftment. I therefore think it is not all that far-fetched.
Kolb: Could you elaborate on the rate of rejection in the unrelated DL-A identical dogs? Secondly, what was the rate of GVH disease? You showed a slide with about 50% rejection and finally you stated that most of these dogs died of GVH. Can you tell how far these dogs were serologically identical and whether you had family studies on this.
Storb: All these dogs are off-springs of large families and characterized by family studies. In the first experiment where we used bone marrow only, $3–4 \times 10^8$ cells/kg, we saw rejection. Of the few dogs which engrafted a number in fact died of GVH. In the second experiment we used marrow plus peripheral blood leukocytes and methotrexate which explains the differences.

Collection, Cryopreservation and Transplantation of Blood Stem Cells in the Treatment of Hemopoietic Failure*

T. M. Fliedner

A. Introduction

It is the purpose of this paper to review the experimental studies which have been carried out in our group to explore the possibilities and limitations to treat hemopoietic failure induced by whole body x-irradiation by means of blood derived hemopoietic stem cells under autologous and allogeneic conditions. Some of the results have been published or are accepted for publication [1, 2, 5–17, 21]. The findings can be summarized in 5 statements supported by sufficient experimental evidence.

B. There Are, in Dogs, Pluripotent Hemopoietic Stem Cells Among the Mononuclear Blood Leukocytes Which Can be Monitored by the Granulocytic Progenitor Cell Culture Method

Evidence for this statement comes from studies in dogs in which it was possible to show that one can collect from the peripheral blood by means of a 4–5 hour continuous flow cell separation procedure ("leukocytapheresis") enough mononuclear leukocytes to restore an aplastic bone marrow after 1200 rd whole body x-irradiation [13].

In these studies, it was demonstrated that the transfusion of 3 to 31×10^9 fresh mononuclear leukocytes results in clearant signs of bone marrow recovery within 10 days. Secondly, the same results were obtained when frozen and thawed cells were used. Mononuclear blood leukocyte suspensions were frozen in a suspension containing 10% DMSO at a controlled cooling rate of 1° C per minute and stored for varying periods of time at ultralow temperatures. They were rapidly thawed before transfusion [6]. The use of cryopreserved cells allowed one to examine the relationship between the number of mononuclear leukocytes transfused into irradiated recipient dogs and the degree of bone marrow and blood cell regeneration. It was found that the extend of hemopoietic bone marrow regeneration is clearly related to the number of mononuclear leukocytes transfused and more than that, to the number of colony forming units in agar (CFU-C) that are among the leukocytes transfused. On day 10 after the transfusion of 2.6×10^9 mononuclear leukocytes, among them 0.8×10^5 CFU-C,

* Research work supported by the Deutsche Forschungsgemeinschaft, the Bundesministerium für Forschung und Technologie, and the European Atomic Community (EURATOM)

one can find about 50% of the marrow niches recovered as far as cellularity is concerned as compared to normal. In the peripheral blood, the number of granulocytes is then back to about 8% of normal [6, 8]. In addition, extensive studies of the lymphatic organs showed that the transfusion of mononuclear blood leukocytes results also in a cell dose dependent regeneration of lymphocytopoiesis in lymphnodes and spleen [21]. Thus, it was concluded that – in dogs – the blood contains mononuclear leukocytes that are able to restore hemopoiesis in bone marrow and lymphatic organs and hence must be considered as "pluripotent" and that the granulocytic progenitor cell culture technique may serve as an indicator for the presence of hemopoietic stem cells [5, 9]. Further support for this statement came from albumin gradient separation studies which clearly indicated a strong correlation between the number of CFU-C in a cell suspension and its hemopoietic repopulation ability and not with the number of mononuclear cells in general [15, 16]. The minimum number of CFU-C that ought to be present in a cell suspension in order to obtain hemopoietic restauration in a lethally irradiated dog is between 20,000 and 40,000 per kg body weight.

C. The Transfusion of Mononuclear Blood Leukocytes and Among them Hemopoietic Stem Cells Results in a Permanent Regeneration of the Blood Cell Forming Organs

It was of particular importance to obtain experimental evidence for this statement since experimental studies in mice had lead to the suggestion that blood derived hemopoietic stem cells may be of limited value because of their limited replicative capability [19]. If blood derived stem cells are to be used in clinical practice, the capability to attain a permanent engraftment becomes of critical importance.

Therefore, dogs were given 1,200 rd whole body x-irradiation and transfused with autologous or allogeneic mononuclear blood leukocytes collected by leukapheresis and cryopreserved as described before. Whenever possible, the transfusion was made with cells from the opposite sex in order to use the sex-chromosome as a marker. In one study involving 7 dogs using autologous cells, the hemopoietic regeneration was followed for between 259 and 898 days [22]. There was no evidence in these nor in other studies, that the stem cell graft did not result in a permanent hemopoietic restauration. As a matter of fact, in one dog, given allogeneic blood leukocytes and methotrexate for several months, the follow-up was continued for over 3 years and showed regenerated hemopoiesis throughout [23]. The study had to be terminated because of logistic reasons. However, radiation induced late effects develop in a number of organs in dogs given 1,200 rd whole body x-irradiation and transfused with blood derived stem cells. It was shown, that fibrotic lesions occur in several organs, including the bone marrow. While there is a remarkable development of "endosteal" fibrosis in autologously transfused dogs, there is, in addition, in allogeneically transfused dogs a typical osteomyelofibrosis [2]. Although, such radiation induced late effects may well be a limiting factor if one considers to transfer the experimental approach described here to the clinical level, it was nevertheless concluded that, in dogs, the use of blood derived stem cells may well result in a permanent

hemopoietic regeneration if the graft-versus-host reaction can be successfully managed (in the allogeneic situation) or is absent (in the autologous situation).

D. The Use of Blood Derived Stem Cells for the Re-establishment of Blood Cell Formation in the Bone Marrow After Lethal Whole Body Radiation Exposure can be Considered as a Repetition of Certain Stages of Fetal Hemopoietic Development

In classical hematology it was felt that hemopoiesis is derived from stem cells that originate from cells of the reticulo-endothelial system [4, 25]. In recent years, tools were developed in experimental hematology that provided evidence for a very special developmental pattern of hemopoiesis [14, 18]. According to these studies, any of the hematopoietic organs is the result of an "endogenous chimerism", consisting of an organspecific "matrix" and an "engrafted" cell population producing organ specific blood cells. Thus, the fetal liver in a 5 week old human fetus is in a prehemopoietic stage of development and resembles a messenchymal "sponge". 1–2 weeks later, hemopoiesis develops, first intrasinuidal, then throughout the organ. This developmental pattern can be considered to mean that stem cells migrate through the blood to the "liver-anlage" and establish fetal blood cell formation. This pattern has been found experimentally in mice [20]. As far as the bone marrow is concerned, there is in all bones of the skeleton a very similar pattern of development. The marrow cavity is first organized by a vascularized mesenchymal cell population forming a "cellular matrix". There is always a period of about 2 weeks during which the fetal marrow is without active hemopoiesis. It is only then that extrasinusoidal "hemopoietic colonies" develop and within a short time the marrow attains its normal cellularity and structure. It is likely that bone marrow hemopoiesis becomes established as a result of stem cell migration via the blood to the bone marrow matrix that is ready to accept hemopoietic stem cells and allows them to replicate and differentiate [8, 14].

Thus, the use of stem cells in the adult animal to restore an aplastic bone marrow is, in a sense, a repetition of the prenatal hemopoietic development: here and there is a cellular matrix devoid of pluripotent stem cells but ready to support cell replication and cellular differentiation. Here and there stem cells migrate through the blood to reach sites that are ready to accept them and to allow the establishment of a "graft". If blood derived hemopoietic stem cells are used, this appears to be a "physiological" source since throughout prenatal development, there are large numbers of stem cells in the blood [8] and their physical properties may well be of advantage for their migration and seeding of a cellular matrix since – at least in the adult dog – the blood CFU-C population is a small subpopulation of the bone marrow CFU-C population [11, 12].

E. The Granulocytic Progenitor Cells (CFU-C) in Dog Blood Represent a Mobilizable Subpopulation of the Bone Marrow CFU-C Pool that Cannot be Depleted Even After Prolonged Leukapheresis

A 5-hour leukocytapheresis in dogs shows, that about four times as many CFU-C can be collected from the peripheral blood as are in it at the beginning of leukapheresis. Furthermore, such a cell separation procedure during which about 12 blood volumes are passing through the cell separation decreases the number of blood CFU-C to about half of its original value but cannot exhaust it [17]. Even a 24-hour continuus flow cell separation does not result in a complete exhaustion of the blood CFU-C pool. But such a procedure indicates clearly, that the number of mobilizable CFU-C that can be removed from the peripheral blood is in excess of 80 times the number of CFU-C present in the blood stream [7]. These findings were interpreted to mean, that there exists in the organism a pool of CFU-C which must be located extravascularly that is in a dynamic balance and perhaps exchange with the intravascular CFU-C pool. The majority of mobilizable CFU-C are most likely in the bone marrow whereas, in the dog, the spleen contributes only little to the blood CFU-C population [24]. That the equilibrium between the bone marrow and the blood CFU-C pool is controlled by regulatory mechanisms is suggested by the fact, that a prolonged leukapheresis which removed 60–80 times the number of blood CFU-C results in a typical "overshoot" reaction of the blood CFU-C concentration. While the blood CFU-C concentration decreases during leukapheresis to about 10% of normal, there is a rapid return to normal within 2–3 days after its completion with an "overshoot" that reaches about 5–6 times the normal concentration after 3–4 days with a return to normal within about 2 weeks. At the same time, the bone marrow CFU-C pool does not seem to be much affected. However, there is – presently – no convincing way to calculate the total size of the bone marrow CFU-C population. That the balance between blood and bone marrow CFU-C is regulated by factors yet to be described is also suggested by studies in which repeated leukapheresis were carried out every 2–3 days for up to 6 times in the same dog. These studies reveal that the blood CFU-C pool increases from 0.6×10^5 to about 2.6×10^5 at the beginning of the 4^{th} as compared to the 1^{st} leukapheresis. The yield of CFU-C that one can obtain during one leukapheresis can thus be increased by a factor of 3 by repeating leukaphereses at appropriate intervals [17]. Another piece of evidence for a balance between the intra- and extravascular CFU-C pool comes from studies in which dextran-sulfate has been used to increase the number of blood CFU-C. Dextran sulfate (DS) is a synthetic polyanion of strong negative charge that has been used successfully to increase the number of circulating CFU-C. It has been demonstrated that 15 mg/kg body weight of DS increase the number of circulating CFU-C by a factor of about 10, while 10 mg/kg body weight result in an increase of 7-fold as compared to the preinjection level within 3 hours after intravenous injection [26, 27]. Although the mechanisms leading to this increase are yet to be explored, it may well be assumed that such an increase is due to the release of mobilizable CFU-C from extravascular pools. This assumption is supported by recent studies in our group that employed velocity sedimentation profiles of circulating as well as of bone

marrow CFU-C under the influence of dextran sulfate mobilization and after prolonged leukocytapheresis [11, 12]. They show that the blood CFU-C are of the same size before and after DS-mobilization and form a small-size subgroup bone marrow CFU-C population. It is only after prolonged leukapheresis that a larger size CFU-C population is being released into the blood. The results of these studies are interpreted to mean that there is a physiological bone marrow-blood barrier for CFU-C allowing normally only small sized CFU-C to enter the blood and that after prolonged drainage of the marrow CFU-C population even larger CFU-C may pass this barrier.

Thus, it is concluded that the CFU-C population in the blood stream – as an indicator of the presence of pluripotent hemopoietic stem cells – is by no means a sign of "shedding" of numbers of overproduced stem cells but is evidence for a population of cells in balance with extravascular sites and well regulated by factors not well known at the present time.

F. The Legality of Graft-Versus-Host-Disease (GVH) After Transfusion of Allogeneic Blood Mononuclear Leukocytes in DLA Matched, MLC Negative Donor-Recipient Combinations Can be Avoided by the Use of "Purified" CFU-C Cell Suspensions

It was clearly demonstrated that both autologous as well as allogeneic mononuclear blood leukocyte suspensions are able to restore bone marrow function within 10 days after transfusion into 1200 rd whole body x-irradiated dogs [1, 5]. However, if no immuno-suppressive therapy is given in the allogeneic situation, only one out of twelve transplanted recipients will survive. All others will die from a variety of courses, most of them from an acute GVH-disease [5]. However, when methotrexate is used to perform an immunosuppression, then 7 out of 14 dogs not only showed a take but survived for more than 80 days without dying from GVH although showing pertinant signs and symptoms [5]. Therefore, the albumin density gradient method proposed originally for bone marrow cell suspensions by Dicke and van Bekkum [3] was used to attempt a purification of the CFU-C population among mononuclear leukocytes. It was of great interest that a three step procedure (DS-mobilization and continuous flow cell collection, a ficollisopaque gradient elimination of red cells proceeding the cell separation on a discontinuous albumin gradient) resulted in a cell suspension in which – on the average – 1 out of 20 mononuclear leukocytes was a CFU-C as compared to one out of about 15,000 in the normal blood [16]. The transfusion of such a purified CFU-C suspension (fraction 2 of the discontinuous albumin gradient) containing some 220,000 CFU-C per kg body weight resulted in a rapid bone marrow recovery recognizable within 10 days after 1200 rd whole body x-irradiation and transfusion and in a blood cell recovery without clinical signs or symptoms of GVH although in a small rise of the blood transaminase levels about 30 to 40 days after transfusion. In contrast, the transfusion of cell suspensions, containing a sufficient number of CFU-C to obtain, in principle, a hemopoietic engraftment within 10 days but being otherwise rich in

lymphocytes, resulted in the death of the animals with severe signs and symptoms of GVH within 3–6 weeks after transfusion [15, 16].

These results justify the hope that it will become possible to use blood stem cell suspensions for the treatment of hemopoietic failure such as induced by exposure of the organism to ionizing radiation. The possibility to segregate a cell population rich in CFU-C and poor in immune reactive cells and to obtain hemopoietic engraftment with the reconstitution of the immune competence but without lethal GVH in dogs may be used to stimulate research with the goal to repeat such results in man and to explore the mechanisms that are responsible for this type of tolerance.

G. Summary

This paper reviews the progress made in studying the means and ways to collect hemopoietic stem cells from the peripheral blood in a preclinical canine model in order to use them for the treatment of a radiation induced bone marrow aplasia. It was demonstrated that one 4–5 hour leukocytapheresis using the IBM-NIH blood cell separator yields enough mononuclear leukocytes, and among them CFU-C, to restore the hemopoiesis of a 1200 rd whole body x-irradiated dog under autologous as well as allogeneic conditions. The cells can be cryopreserved and stored for months or even years. The expected graft-versus-host reaction even after the transfusion of DLA matched, MLC negative mononuclear leukocytes can be successfully treated in many cases using methotrexate. The GVH-reaction was eliminated by transfusing a leukocyte suspension with a high CFU-C concentration (1 in 20 MNC) obtained by employing the discontinuous albumin density gradient centrifugation method.

References

1. Calvo, W., Fliedner, T. M., Herbst, E., Hügl, E., Bruch, C.: Regeneration of blood forming organs after autologous leukocyte transfusion in lethally irradiated dogs. II. Distribution and cellularity of the marrow in irradiated and transfused animals. Blood *47*, 593–601 (1976)
2. Calvo, W., Fliedner, T. M., Steinbach, I., Alcober, V., Nothdurft, W., Fache, I.: Morphologic alterations in canine marrow of long-term survivors after 1200 R whole-body x-irradiation and autologous blood leukocyte engraftment. Am J. Pathol. *95*, 379–388 (1979)
3. Dicke, K. A., Bekkum, D. W. van: Avoidance of acute secondary disease by purification of hemopoietic stem cells with density gradient centrifugation. Exp. Hematol. *20*, 126–130 (1970)
4. Doan, C. A.: On the origin and developmental potientialities of blood cells. Bull. NY Acad. Med. *15*, 668–697 (1939)
5. Fliedner, T. M., Flad, H.-D., Bruch, C., Calvo, W., Goldmann, S., Herbst, E., Hügl, E., Huget, R., Körbling, M., Krumbacher, K., Nothdurft, W., Ross, W. M., Schnappauf, H.-P., Steinbach, I.: Treatment of aplastic anemia by blood stem cell transfusion: a canine model. Haematologica *61*, 141–156 (1976)
6. Fliedner, T. M., Körbling, M., Calvo, W., Bruch, C., Herbst, E.: Cryopreservation of blood mononuclear leukocytes and stem cells suspended in a large fluid volume. Blut *35*, 195–202 (1977)
7. Fliedner, T. M., Calvo, W., Körbling, M., Kreutzmann, H., Nothdurft, W., Ross, W. M., Vassileva, D.: Hematopoietic stem cells in blood: characteristics and potentials. In: Hematopoie-

tic cell differentiation. Golde, D. W. et al. (eds.), pp. 193–212. New York, San Francisco, London: Academic Press 1978.
8. Fliedner, T. M., Calvo, W.: Hematopoietic stem-cell seeding of a cellular matrix: a principle of initiation and regeneration of hematopoiesis. In: Differentiation of normal and neoplastic hematopoietic cells. Cold Spring Harbor Laboratory 1978
9. Fliedner, T. M., Calvo, W., Körbling, M., Nothdurft, W., Pflieger, H., Ross, W. M.: Collection, storage and transfusion of blood stem cells for the treatment of hemopoietic failure. Blood Cells *5*, 313–328 (1979)
10. Fliedner, T. M., Körbling, M., Arnold, R., Grilli, G., Haen, M., Kreutzmann, H., Pflieger, H.: Collection and cryopreservation of mononuclear blood leukocytes and CFU-C in man. Exp. Hematol. *7*, Suppl. 5, 398–408 (1979)
11. Gerhartz, H. H., Fliedner, T. M.: Granulozytär determinierte Stammzellen (CFU-C) aus Knochenmark und Blut: Größenunterschiede vor und nach Dextransulfat-Mobilisation. Blut *38*, 161–164 (1979)
12. Gerhartz, H. H., Fliedner, T. M.: Velocity sedimentation and cell cycle characteristics of granulopoietic progenitor cells (CFU-C) in canine blood and bone marrow: Influence of mobilization and CFU-C depletion. Exp. Hematol. (accepted for publication)
13. Herbst, W. E., Fliedner, T. M., Calvo, W., Schnappauf, H.-P., Meyer, H.: Untersuchungen über die Gewinnung hämopoetischer Stammzellen aus dem peripheren Blut von Hunden und über ihre Fähigkeit, die Blutzellbildung zu regenerieren. Blut *30*, 265–276 (1975)
14. Kelemen, E., Calvo, W., Fliedner, T. M.: Atlas of human hemopoietic development. Heidelberg, Berlin, New York: Springer 1979
15. Körbling, M., Fliedner, T. M., Calvo, W., Nothdurft, W., Ross, W. M.: In-vitro and in-vivo properties of canince blood mononuclear leukocytes separated by discontinuous albumin density gradient centrifugation. Biomedicine *26*, 275–283 (1977)
16. Körbling, M., Fliedner, T. M., Nothdurft, W., Calvo, W., Ross, W. M., Steinbach, I.: Albumin density purification of canine hemopoietic blood stem cells (HBSC): Long-term allogeneic engraftment without gvh-reaction. Exp. Hematol. accepted for publication (1979)
17. Kovács, P., Bruch, C., Herbst, E. W., Fliedner, T. M.: Collection of in vitro colony-forming units from dogs by repeated continuous flow leukaphereses. Acta Haematol. *60*, 172–181 (1978)
18. Metcalf, D., Moore, M. A. S.: Hemopoietic cells. Amsterdam, London: North-Holland Publishing Company 1971
19. Micklem, H. S., Ogden, D. A., Evans, E. P., Ford, C. E., Gray, I. G.: Compartments and cell flows within the mouse haemopoietic system. II. Estimated rates of interchange. Cell Tissue Kinet. *8*, 233–248 (1975)
20. Moore, M. A. S., Metcalf, D.: Ontogeny of the hemopoietic system: Yolk sac origin of in-vivo and in-vitro colony forming cells in the developing mouse embryo. Br. J. Haematol. *18*, 279–283 (1970)
21. Nelson, B., Calvo, W., Fliedner, T. M., Herbst, E., Bruch, C., Schnappauf, H.-P., Flad, H.-D.: The repopulation of lymph nodes of dogs after 1200 R whole-body x-irradiation and intravenous administration of mononuclear blood leukocytes. Am. J. Pathol. *84*, 259–278 (1976)
22. Nothdurft, W., Bruch, C., Fliedner, T. M., Rüber, E.: Studies on the regeneration of the CFU-C population in blood and bone marrow of lethally irradiated dogs after autologous transfusion of cryopreserved mononuclear blood cells. Scand. J. Haematol. *19*, 470–481 (1977)
23. Nothdurft, W., Fliedner, T. M., Calvo, W., Flad, H.-D., Huget, R., Körbling, M., Krumbacher von Loringhofen, K., Ross, W. M., Schnappauf, H.-P., Steinbach, I.: CFU-C populations in blood and bone marrow of dogs after lethal irradiation and allogeneic transfusion with cryopreserved blood mononuclear cells. Scand. J. Haematol. *21*, 115–130 (1978)
24. Nothdurft, W., et al.: Unpublished observations
25. Rohr, K.: Das menschliche Knochenmark. Stuttgart: Thieme 1960
26. Ross, W. M., Körbling, M., Nothdurft, W., Calvo, W., Fliedner, T. M.: Characterization of bone marrow and lymph node repopulating cells by transplanting mononuclear cells into radiated dogs. In: Experimental hematology today. Baum, S. J., Ledney, G. D. (eds.) pp. 29–38. New York: Springer 1977
27. Ross, W. M., Körbling, M., Nothdurft, W., Fliedner, T. M.: The role of dextran sulfate in increasing the CFU-C concentration in dog blood. Proc. Soc. Exp. Biol. Med. *157*, 301–305 (1978)

Discussion

Dicke: What is the minimal number of mononuclear cells from the peripheral blood to get engraftment?
Fliedner: If we relate it to mononuclear cells it is in the order of 3×10^9. I think to relate it to CFU-C is more relevant because if you do several leukaphereses you have a higher yield of CFU-C than in the first.
Dicke: What is the minimal number of leukaphereses for engraftment?
Fliedner: In the dog we get a comfortable and safe engraftment with 3.2×10^9 mononuclear cells and 220,000/kg CFU-C.
Dicke: So you process 4–5 litres of blood of the dog with leukapheresis.
Fliedner: We run about 12 litres during 4 hours.
Prindull: Under steady state condition do you have any data how long the CFU-C circulate in the blood before they leave it?
Fliedner: We would like to know because this is a very important question. Kommatsch has calculated from our leukapheresis studies that there may be a transit time through the blood of about 20 minutes. I dont know whether this has any meaning because the fate of the circulating stem cell is unknown. I agree with Micklem who thinks that under normal steady state conditions when all stem cell niches are filled up there may not be a need for reseeding.

Bone Marrow Transplantation in DLA-Haploidentical Canine Littermates
Fractionated Total Body Irradiation (FTBI) and in Vitro Treatment of the Marrow Graft with Anti-T-Cell Globulin (ATCG)

H. J. Kolb, U. Bodenberger, H. V. Rodt, I. Rieder, B. Netzel, H. Grosse-Wilde, S. Scholz and S. Thierfelder

A. Introduction

Allogeneic bone marrow transplantation requires tolerance of the host versus the graft as well as tolerance of the graft versus the host. This mutual tolerance can be induced in most instances of marrow transplantation between DLA-identical siblings by conditioning the host with total body irradiation (TBI) or Cyclophosphamide and post grafting treatment with Methotrexate [1].

These methods are not adequate for induction of lasting tolerance across major histocompatibility differences [2]. Marrow grafts from histoincompatible family members were significantly less successful than those from DLA-matched littermates, but somewhat better than those of histoincompatible unrelated donors [3].

A treatment regimen which allows marrow grafts between one haplotype different family members would provide possibilities of marrow transplantation for almost every patient lacking an HLA-identical sibling. In the present study bone marrow was grafted between canine littermates which differed in one DLA-haplotype. We investigated, whether fractionated total body irradiation with high total doses can suppress the hosts immune reaction against the DLA-incompatible graft and whether in vitro treatment of the graft with an anti-T-cell gobulin from which crossreacting antibodies against hemopoietic stem cells have been absorbed can suppress the grafts reaction against a DLA-incompatible host.

B. Materials and Methods

I. Dogs

The dogs used for transplantation studies were beagles bred in the kennels of the Gesellschaft für Strahlen- und Umweltforschung. Their ancestors were acquired from five different commercial breeding colonies in Europe. The dogs weighing 8 to 15 kg were vaccinated against distemper, canine hepatitis and leptospirosis, regularly dewormed, and at least 6 months old. Absorption material was obtained from mongrel dogs.

II. DLA-Testing

Antigens of the DLA-A and -B locus were determined with the microlymphocytotoxicity test [4, 5], those of the DLA-D locus were defined in mixed leukocyte culture (MLC) with DLA-D homozygous typing cells as previously described [6]. DLA-haplotypes were defined by segregation analysis. DLA-homozygous donors and DLA-heterozygous littermate recipients sharing the DLA-haplotype

of the donor were selected for graft-versus-host disease (GVHD) experiments. In these combinations mixed leukocyte cultures (MLC) regularly showed the response of the donor against the host and no response of the host against the donor.

DLA-heterozygous littermates sharing one DLA-haplotype und differing in the second haplotype showed mutual MLC responses and were selected for experiments involving host versus graft and graft versus host reactions.

III. Total Body Irradiation

Total body irradiation was applied from two opposing ^{60}Co-surces to the dog placed in a wooden cage midway between the sources. The cage had an interior width of 55 × 97 cm and a hight of 44 cm in which the dog could move around. The dose rate was 5.5 R/min delivered at a source target distance of 400 cm. The single dose of TBI consisted of 1200 R given on day −1, fractionated total body irradiation (FTBI) was given as 1200 R on day −1 and additional fractions of 600 R on days −7 and/or −5 and −3. 600 R were chosen as a large dose fraction without clinical symptoms of gastrointestinal toxicity. Dogs were kept off food and water on the days of irradiation and for five days after the last irradiation. They were given parenteral fluids twice daily and antibiotics as clinically indicated. Marrow cells were given 24 hours after the last irradiation.

IV. Bone Marrow Transplantation

Bone marrow was obtained from the anaesthetized donor by aspiration from humeri, femora and pelvic crest. It was mixed with tissue culture medium TC 199 containing heparin and processed as described by Thomas and Storb [7]. In cases of "in vitro" treatment 200 ml bone marrow was mixed with one or two ml absorbed ATG diluted in 10 ml TC 199 and incubated at 4° C for 30 min with constant shaking. Thereafter it was infused into the recipients within 10 to 20 min. Hemopoietic recovery was evident by a rise of blood counts following the postirradiation nadir (granulocytes of more than 350/mm^3, platelets more than 20,000/mm^3). GVHD was diagnosed clinically by the typical skin rash, jaundice and diarrhea. It was confirmed with complete post mortem examination including histology.

Proof of chimerism was obtained by cytogenetic analyses and DLA-typing. Karyotypes were analysed in direct 3-hour cultures of bone marrow and in 3-day cultures of blood lymphocytes stimulated with phytohemagglutinin.

V. Production, Absorption and Testing of Antithymocyte Globulin

Antithymocyte serum was produced by i.v. injection of 10^8 thymocytes of new born dogs into rabbits followed by three daily booster injections three weeks later and exsanguination four weeks later.

Rabbit anti-dog thymocyte globulin was absorbed with 2 × liver/kidney homogenate, 3 × newborn spleen, 2 × erythrocytes at 4° C for 30 min at a weight ration of sediment: antiserum of 1:4. The antiserum was purified as globulin fraction by ammonium sulfate precipitation, DEAE ion exchange chromatography and ultracentrifugation [8]. ATG was reconcentrated to 10 mg/ml.

Two antisera were produced and tested in complement fixation tests before and after absorption. The titer against marrow cells was selectively lowered in both (Table 1). The remaining titer of 5 and 7,5 may in part be caused by admixture of blood in the aspirated marrow sample.

4×10^6 mononuclear cells were incubated with the antibody preparation in a final volume of 0.5 ml of TC 199 medium for 30 min at 4° C. Fresh dog serum was added as the source of complement (25% final dilution) and the suspension further incubated for 45 min at 37° C. The cells were then washed once and 10^5 mononuclear marrow cells were plated in semisolid agar (0.3%). As a source of colony stimulating activity 20% serum from dogs irradiated and bled 10 days later was added. Colonies (defined as groups of 50 and more cells) were counted after a culture period of 8 days.

The unabsorbed antisera inhibited colony growth up to titers higher than 1:32 and 1:128, while absorbed antisera did not inhibit colony growth at titers of 1:8 and 1:128 (Table 2).

VI. Cryopreservation of Bone Marrow

The cryopreservation of bone marrow was performed as described by Buckner et al. [10]. Aspirated marrow was given in 50 ml portions into 600 ml Travenol-Fenwal bags mixed with 50 ml tissue culture

Table 1. Complement fixation of unabsorbed and absorbed anti-thymocyte globulin

Antiserum-No.	Log-2-titer with 50% hemolysis against		
	Thymus	Blood lymphocytes	Marrow
620 unabsorbed	9.5	8.5	9
618 absorbed	7.5	7	5
741 unabsorbed	10.5	9	9.5
819 absorbed	10.5	7.9	7.5

Marrow was obtained by aspiration

Table 2. CFU-C growth in marrow following incubation with unabsorbed and absorbed ATG

Antiserum-No.	No. of colonies/10^5 mononuclear bone marrow cells at concentration ATG/marrow of				
	1:8	1:16	1:32	1:64	1:128
620 unabsorbed	–	3±1	7±3	–	–
618 absorbed	337±11	–	335±5	–	–
741 unabsorbed	0	0	0	3	6
819 absorbed	0	0	0	5	320

Normal rabbit globulin 298±14

medium TC 199 containing 20% dimethylsulfoxide (DMSO) and frozen at a rate of 1° C per minute to −20° C, then at a rate of 4° C per minute to −40° C and finally at a rate of 8° C per minute to −80° C. It was stored in the vapour phase of liquid nitrogen. The thawing procedure included rapid thawing in a 40° C water bath, dilution of the marrow with an equal volume TC 199 containing 20% dog plasma with 15% ACD, centrifugation, removal of supernatant and immediate infusion.

VII. Methotrexate Treatment After Grafting

Methotrexate was given i.v. in doses of 0.25 mg/kg body weight on days 1,3 and 6 and of 0.5 mg/kg weekly from day 11 until day 102 as described by Storb et al. [9].

Table 3. Autologous transplantation of marrow with and without in vitro treatment with absorbed ATG

Dog. No.	ATG-treatment-concentration vol ATG: vol marrow	No. of marrow cells infused ($\times 10^8$/kg BW)	Day of recovery of Leukocytes (>1000/mm^3)	Platelets (>20,000/mm^3)
D 42	–	2.8	7	13
D 43	–	4.8	9	15
D 39	–	5.0	9	11
D 36	–	8.0	11	20
N 446	1:100	4.0	8	11
L 372	1:50	2.8	11	16
N 448	1:25	2.9	12	21

Table 4. Results of bone marrow transplantation in DLA-incompatible littermate dogs with and without in vitro treatment of the marrow with absorbed ATG

D (onor) R (eci-pient)	No.	Sex	DLA-type D-A-B	ATG-treatment Vol. ATG: vol. marrow	No. of marrow cells transf. × 10^8/kg body weight	Survival time (days)	Outcome
Without ATG treatment							
D-N	178	♂	50-2-4/50-2-4				
R-N	177	♀	50-2-4/56-1-13	–	6.2	16	death with GVHD
D-N	178	♂	50-2-4/50-2-4				
R-N	180	♀	50-2-4/56-1-13	–	3.8	17	death with GVHD
D-N	152	♂	50-2-4/50-2-4				
R-N	153	♀	50-2-4/52-9-6	–	1.9	22	death with GVHD
D-N	418	♂	50-2-4/50-2-4				
R-N	416	♀	50-2-4/52-9-6	–	3.0	23	death with GVHD
D-N	176	♂	50-2-4/50-2-4				
R-N	173	♀	50-2-4/53-3-12	–	5.5	27	death with GVHD
ATG treatment with No. 618							
D-N	332	♂	56-1-13/56-1-13				death without sustained
R-N	333	♂	56-1-13/53-3-12	1:100	2.6	16	hemopoietic recovery
D-N	353	♂	50-2-4/50-2-4				death without sustained
R-N	351	♀	50-2-4/52-9-6	1:100	7.0	36	hemopoietic recovery
D-N	375	♂	51-2-5/51-2-5				
R-N	374	♀	51-2-5/56-1-13	1:100	2.3	61	death with GVHD
D-N	323	♂	50-2-4/50-2-4				
R-N	321	♀	50-2-4/56-1-13	1:100	7.7	70	death with GVHD
D-N	384	♂	51-2-5/51-2-5				
R-N	380	♀	51-2-5/56-1-13	1:100	4.7	>21 months	alive without GVHD
D-N	323	♂	50-2-4/50-2-4				
R-N	328	♀	50-2-4/52-9-6	1:200	4.9	29	death with GVHD
D-N	353	♂	50-2-4/50-2-4				
R-N	354	♂	50-2-4/52-9-6	1:200	3.1	66	death with GVHD

D-N	417	♂	50-2-4/50-2-4				
R-N	415	♀	50-2-4/52-9-6	1:200	3.9	>18 months	alive without GVHD
D-N	384	♂	51-2-5/51-2-5				
R-N	383	♂	51-2-5/56-1-13	1:200	2.2	>23 months	alive without GVHD
D-N	381	♀	51-2-5/51-2-5	1:200			
R-N	382	♀	51-2-5/56-1-13	1:200	2.4	>23 months	alive without GVHD
ATCG treatment with No. 819							
D-N	555	♂	50-2-4/50-2-4				
R-N	552	♀	50-2-4/56-1-13	1:800	7.0	25	death with GVHD
D-N	555	♂	50-2-4/50-2-4				
R-N	554	♂	50-2-4/52-9-6	1:400	4.8	32	death with GVHD
D-N	558	♀	50-2-4/50-2-4				
R-N	562	♂	50-2-4/52-9-6	1:400	5.8	40	death with GVHD
D-N	465	♂	51-2-5/51-2-5				
R-N	464	♀	51-2-5/56-1-13	1:200	5.3	>79	living, no GVHD
D-N	465	♂	51-2-5/51-2-5				
R-N	462	♀	51-2-5/56-1-13	1:200	7.2	>102	living, no GVHD
D-N	558	♀	50-2-4/50-2-4				
R-N	559	♀	50-2-4/52-9-6	1:200	7.7	>114	living, no GVHD
D-N	570	♀	56-1-13/56-1-13				
R-N	572	♂	56-1-13[a]/53-3-12	1:100	1.0	21	rejection of the graft
D-N	496	♂	50-2-4/50-2-4				
R-N	494	♀	50-2-4/bl-9-bl	1:100	8.3	44	death with GVHD
D-N	496	♂	50-2-4/50-2-4				
R-N	491	♀	50-2-4/bl-9-bl	1:100	5.7	>77	living, no GVHD

[a] DLA type serologically not unequivocal, MLC result unequivocal

C. Results

I. In Vitro Treatment of the Marrow Graft wit ATCG

Autologous bone marrow transplantation was studied following 1200 R TBI with and without incubation of the marrow with ATCG. Even at the highest concentration of ATCG (1:25) used reconstitution of hemopoiesis was complete and recovery of leukocyte and platelet counts was not delayed.

Allogeneic marrow was grafted from DLA-homozygous littermates into DLA-heterozygous – one haplotype different – recipients. The marrow was either incubated with one of two ATCG-preparations or untreated. Dogs given the untreated marrow died within four weeks with severe GVHD (Table 4). The first preparation (No. 618) was used at concentrations of 1:100 and 1:200. Two dogs died without complete hematologic recovery, in four dogs GVHD was modified and in four a lasting graft-versus-host tolerance was induced. The results with the recent batch (No. 819) were comparable. At concentrations of 1:100 and 1:200 four dogs developed lasting graft-versus-host tolerance, one dog died with delayed GVHD and one dog with marrow aplasia following rejection of the graft. The incubation with lower concentrations (1:400 and 1:800) only delayed the onset of GVHD. Chimerism was studied in five survivors by karyotype analyses of marrow and blood cells and in three by DLA-typing including mixed leukocyte culture. In all instances chimerism was complete (Table 5).

Table 5. Evidence of chimerism in survivors

Dog No.	Day post grafting	No. of donor karyotypes/ karyotypes evaluated		DLA-type post grafting
		Marrow	Blood	
N 464	30	20/20	20/20	–
N 491	28	20/20	19/20	50-2-4[a]
N 462	28	20/20	–	–
	41	–	20/20	51-2-5[a]
N 559	105	–	–	50-2-4[a]
N 415	23	20/20	–	–
	50	3/3	20/20	–
	97	20/20	20/20	–
	17 months		20/20	50-2-4[a]
N 380	31	–	16/16	–
	46	–	19/19	–
	59	2/2	–	–
	150	6/6	20/20	51-2-5[a]
	20 months	20/20	20/20	51-2-5[a]
N 382	150	–	–	51-2-5[a]
	23 months	–	–	51-2-5[a]
N 383	150	–	–	51-2-5[a]
	23 months	–	–	51-2-5[a]

[a] The chimeras cells did not stimulate the donors cells in mixed leukocyte culture

II. Fractionated Total Body Irradiation

In autologous marrow grafting studies a regimen of FTBI was worked out that allowed total body irradiation with 3000 R. Fractions of 600 R were added to the standard single dose of 1200 R described by the Seattle group [1], since single doses of 2400 R were not tolerated by all dogs even at the lowest dose rate of 0.5 R/min (Table 6). Daily irradiation was not well tolerated, whereas irradiation every other or every third day was tolerated. FTBI with 3600 R produced severe toxicity in the alimentary tract, 3000 R were well tolerated.

Table 6. Fractionated total body irradiation and marrow infusion

Total dose (R)	Schedule (days before marrow infusion) −9 −8 −7 −6 −5 −4 −3 −2 −1	No. of dogs surviving 30 days/No. of dogs studied
2,400		2/7
2,400		5/8
2,400		1/4
2,400		4/4
2,400		3/4
2,400		6/6
3,000		3/3
3,600		0/4

■ 5,5 R/min. □0,5 R/min.

Allogeneic marrow grafts were carried out between DLA-heterozygous littermates which were mismatched in one DLA-haplotype. One group of six dogs was given a single dose of 1200 R TBI and a hemopoietic graft of marrow and peripheral blood leukocytes (Table 7). Three dogs rejected the graft and died with marrow aplasia, three dogs showed engraftment and died with severe GVHD. Marrow without peripheral blood leukocytes was given to dogs conditioned with FTBI (Table 8). Marrow engraftment was observed after 3000 R and 2400 R FTBI. After 3000 R dermatitis on head and limbs and mucositis in nose and mouth was particularly severe indicating radiation induced changes in addition to GVHD. Modification of GVHD was attempted by in vitro treatment of the marrow with ATCG and by methotrexate treatment after grafting. Evidence of engraftment was seen in all dogs, but two dogs given in vitro treated marrow died early with only slight rises in white blood counts. Four dogs given methotrexate developed severe GVHD and died between 20 and 53 days after grafting. Two dogs given in vitro treated marrow and methotrexate showed hemopoietic engraftment without developing GVHD, but suffered of wasting. One of these died with epileptic seizures, the other of wasting.

D. Discussion

In the majority of cases, bone marrow transplantation between histocompatible canine family members unlike DLA-matched littermates is unsuccessful [2].

Table 7. Single dose total body irradiation with 1200 R and hemopoietic grafts from DLA-incompatible (one haplotype different) littermates

Dog-No.	Sex	DLA-D-A-B	Marrow cells $\times 10^8$/kg	Buffy coat $\times 10^8$/kg	Survival time (days)	Outcome
D-N 407	♀	50-2-4/55-3-bl				rejection, death
R-N 412	♂	50-2-4/52-9-6	4.0	7.0	10	with marrow aplasia
D-N 308	♂	52-9-6/56-1-13				
R-N 309	♂	52-9-6/50-2-4	5.9	10.9	13	take, death with GVHD
D-N 369	♂	50-2-4/56-1-13				
R-N 372	♀	50-2-4/53-3-12	2.7	7.5	13	take, death with GVHD
D-N 408	♀	50-2-4/55-3-bl				rejection, death
R-N 413	♂	50-2-4/52-9-6	4.7	6.2	14	with marrow aplasia
D-N 408	♀	50-2-4/55-3-bl				rejection, death
R-N 414	♂	50-2-4/52-9-6	2.1	4.5	15	with marrow aplasia
D-N 262	♂	51-2-5/50-2-4				
R-N 263	♀	51-2-5/52-9-6	6.5	11.8	17	take, death with GVHD

However survival after marrow grafts from family members is better than that after marrow grafts from unrelated dogs. Most histoincompatible family members differ by one DLA-haplotype and share the second haptlotype. Also, marrow graft from DLA-haploidentical littermates following conditioning treatment with cyclophosphamide [11, 12] or total body irradiation [13] have been unsuccessful because of rejection of the marrow graft or fatal GVHD. In the present study as in one previously reported [14], in vitro treatment of the marrow graft with ATCG prevented acute and chronic GVHD in about half of the dogs of a donor recipient combination with a relatively weak host versus graft reaction. These results have been reproduced with a second batch ATCG which had been obtained and absorbed independently from the first batch. These aminals survive presently as stable chimeras for more than two years. These results indicate that in vitro treatment of the marrow with ATCG is a superior method for prevention of GVHD than treatment with methotrexate after grafting [13].

The ATCG preparations were effective at concentrations of 1:200 and 1:100 at which colony growth in agar (Table 2) and hemopoietic recovery following autologous transplantation was not suppressed. However, in three dogs hemopoietic reconstitution by allogeneic transplantation was prevented and in three cases of in vitro treatment with the second batch ATCG at lower concentrations GVHD was not prevented. It is therefore not possible to conclude from complement fixing titer of ATCG which concentration of ATCG is best for application in vivo. A similar approach for the prevention of GVHD with in vitro treatment with anti-T-cell globulin was successful in mice [15] and chickens [16], it was less successful in rats [17] in which only an antilymphocyte globulin with broader specificity could prevent GVHD. The most likely effect of in vitro treatment with ATCG on GVHD is the elimination of T-cells responsible for graft-versus-host reactions. Thereby, hemopoietic stem cells could be preserved by absorption of crossreacting antibodies from the serum.

Table 8. Fractionated total body irradiation and marrow grafts from DLA-haploidentical littermates

D(onor) R(eci-pient)	No.	Sex	DLA-type D-A-B	Dose in R	No. of marrow cells transf. 10^8/kg b.w.	ATG-treatment 1:400	MTX post graft	Survival time (days)	Outcome
D-N	576	♂	51-2-5/52-9-6						
R-N	574	♀	51-2-5/56-1-13	3,000	6.9	−	−	12	take and death with GVHD
D-N	455	♀	50-2-4/56-1-13						
R-N	457	♀	50-2-4/53-3-12	3,000	5.7	−	−	13	take and death with GVHD
D-N	527	♂	53-3-12/52-9-6						
R-N	521	♀	53-3-12/56-1-13	2,400	4.5	−	−	10	take and death with infection
D-N	540	♂	bl-3-6/50-2-4						
R-N	534	♀	bl-3-6/52-9-6	2,400	6.2	−	−	20	take and death with GVHD
D-N	526	♂	56-1-13/52-9-6						
R-N	525	♂	56-1-13/53-3-12	2,400	3.7	−	+	28	take and severe GVHD
D-N	568	♂	51-2-5/57-8-13						
R-N	567	♂	51-2-5/56-1-13	2,400	5.4	−	+	20	take and death with GVHD
D-N	577	♂	52-9-6/51-2-5						
R-N	576	♂	52-9-6/56-1-13	2,400	6.7	−	+	24	take and death with GVHD
D-N	567	♂	51-2-5/56-1-13						
R-N	564	♀	51-2-5/57-8-13	2,400	5.4	−	+	53	take, killed because of severe GVHD
D-N	569	♂	51-2-5/56-1-13						
R-N	566	♂	51-2-5/57-8-13	2,400	8.1	+	+	8	take (?) and death with infection
D-N	527	♂	52-9-6/53-3-12						
R-N	523	♀	52-9-6/56-1-13	2,400	6.7	+	+	9	take (?) and death with infection
D-N	507	♂	53-3-12/52-9-6						
R-N	506	♀	53-3-12/51-2-5	2,400	6.1	+	+	30	take and death with severe wasting
D-N	566	♂	51-122-5/57-8-13						
R-N	563	♀	51-2-5/56-1-13	2,400	5.9	+	+	39	take and death with epileptic seizures

Evidence of chimerism by donor karyotypes:

N 564 blood: 20/20 marrow: 5/5 (day 25) N 506 marrow: 11/11 (day 19) N 563 blood: 6/6 (day 24)

However, donor T-cells contained in peripheral blood leukocytes have been shown to enhance chimerism in marrow grafted dogs conditioned with cyclophosphamide [18] and engraftment of DLA-incompatible marrow in dogs conditioned with TBI [19]. This favorable effect of T-cells can not be used, if at the same time GVHD should be prevented by elimination of T-cells from the marrow. Another method of stronger suppression of the host-versus-reaction was found in FTBI with high total doses. The results of FTBI compare favorably with those obtained by adding peripheral blood leukocytes [19] and "total lymphoid irradiation" [20], since engraftment was successful in all cases and complete chimerism could be demonstrated with karyotype analyses.

Furthermore, FTBI with high total doses may be preferred because of its better antileukemic effect. Methotrexate given after grafting has been shown to improve survival by modification of GVHD in mice a21] and dogs [22]. In the present study, GVHD was not prevented by methotrexate. In vitro treatment of the graft with ATCG in addition to methotrexate prevented GVHD, but severe infections and wasting are remaining problems. These problems may either be related to severe immune deficiency following FTBI and infusion of a T-cell free marrow or to persisting host-versus-graft reactivity which hinderes the grafts function.

However, these preliminary results promise that FTBI and in vitro treatment of the marrow graft with ATCG may pave the way for bone marrow transplantation between haploidentical family members. In this case, a marrow donor would be available for almost every patient in need of bone marrow transplantation.

References

1. Storb, R., Rudolph, R. H., Kolb, H. J., Graham, T. C., Mickelson, E., Erickson, V., Lerner, K. G., Kolb, H., Thomas, E. D.: Marrow grafts between DLA-matched canine littermates. Transplantation *15,* 92–100 (1973)
2. Storb, R., Epstein, R. B., Bryant, J., Ragde, H., Thomas, E. D.: Marrow grafts by combined marrow and leukocyte infusions in unrelated dogs selected by histocompatibility typing. Transplantation *6,* 587–593 (1969)
3. Storb, R., Kolb, H. J., Graham, T. C., Leblond, R., Kolb, H., Lerner, K. G., Thomas, E. D.: Marrow grafts between histoincompatible canine family members. Rev. Europ. Etudes Clin. et Biol. *17,* 680–685 (1972)
4. Vriesendorp, H. M., Epstein, R. B., Amaro, J. D.: Transplantation *14,* 299 (1972)
5. Terasaki, P. E., McCleland, J. D.: Nature *204,* 998 (1964)
6. Grosse-Wilde, H., Vriesendorp, H. M., Netzel, B., Mempel, W., Kolb, H. J., Wank, R., Thierfelder, S., Albert, E. D.: Transplant. Proc. *7,* 159 (1975)
7. Thomas, E. D., Storb, R.: Blood *36,* 507 (1970)
8. Rodt, H., Thierfelder, S., Thiel, E., Götze, D., Netzel, B., Huhn, D., Eulitz, M.: Immunogenetics *2,* 411 (1975)
9. Storb, R., Epstein, R. B., Graham, T. C., Thomas, E. D.: Methotrexate regimens for control of graft-versus-host disease in dogs with allogeneic marrow grafts. Transplantation *9,* 240–246 (1970)
10. Buckner, C. D., Storb, R., Dillingham, L. A., Thomas, E. D.: Cryobiology *7,* 136 (1970)
11. Kolb, H. J., Rieder, I., Grosse-Wilde, H., Scholz, S., Kolb, H., Wallner, B., Netzel, B., Albert, E. D., Thierfelder, S.: Canine marrow grafts in donor-recipient combinations with one-way nonstimulation in mixed lymphocyte culture. Transplant. Proc. *7,* 461–464 (1975)

12. Kolb, H. J., Rieder, I., Grosse-Wilde, H., Abb, J., Albert, E., Kolb, H., Schäffer, E., Thierfelder, S.: Marrow grafts in LD-SD typed dogs treated with Cyclophosphamide. Transplant. Proc. *8*, 555–559 (1976)
13. Storb, R., Weiden, P. L., Schoeder, M. L., Graham, T. C., Lerner, K. G., Thomas, E. D.: Marrow grafts between canine littermates homozygous or heterozygous for lymphocyte-defined histocompatibility antigens. Transpantation *21*, 299–306 (1976)
14. Kolb, H. J., Rieder, I., Rodt, H., Netzel, B., Grosse-Wilde, H., Scholz, S., Schäffer, E., Kolb, H., Thierfelder, S.: Anti lymphocytic antibodies and marrow transplantation. VI. Graft-versus-host tolerance in DLA-incompatible dogs following "in vitro" treatment of bone marrow with absorbed anti thymocyte globulin. Transplantation (in press)
15. Rodt, H., Thierfelder, S., Eulitz, M.: Exp. Hematol. *2*, 195ff. 1974
16. Ivanyi, J.: Prevention of graft-versus-host reactions and conditioning of recipients for bone marrow transplantation in chickens. In: Recent trends in the Immunology of bone marrow transplantation. Thierfelder, S. et al. (eds.). Berlin, Heidelberg, New York: Springer 1980
17. Müller-Ruchholtz, W., Wottge, H.-U., Müller-Hermelink H. R.: Restitution potentials of allogeically or xenogenically grafted lymphocyte-free hemopoietic stem cells. In: Recent trends in the immunology of bone marrow transplantation. Thierfelder, S. et al. (eds.). Berlin, Heidelberg, New York: Springer 1980
18. Rieder, I., Kolb, H. J., Schäffer, E., Kolb, H., Grosse-Wilde, H., Scholz, S., Thierfelder, S.: Leukocyte transfusions for the modification of host-versus-graft reactions in dogs. In: Experimental hematology today. Baum, S. J., Ledney, G. D., (eds.) p. 101–107. Springer 1978
19. Weiden, P. L., Storb, R., Graham, T. C., Sale, G. E., Thomas, E. D.: Resistance to DLA-nonidentical marrow grafts in lethally irradiated dogs. Transplant. Proc. *4*, 285–288 (1977)
20. Slavin, S., Reitz, B. A., Bieber, C. P., Hoppe, R., Fuks, Z., Gottlieb, M., Grunnet, C., Kaplan, H. S., Strober, S.: Establishment of permanent and specific transplantation tolerance to bone marrow and organ allografts across major histocompatibility barriers. Transplant. Proc. (in press)
21. Uphoff, D. E.: Alteration of hemograft reaction by Amethoptein in lethally irradiated mice treated with homologous marrow. Proc. Soc. Exp. Biol. Med. *99*, 651 (1958)
22. Thomas, E. D., Collins, J. A., Herman, E. C., Ferrebec, J. W.: Marrow transplants in lethally irradiated dogs given Methotrexate. Blood *19*, 217–228 (1962)

Discussion

Vriesendorp: I think the system you showed in which you did an autologous transplant with ALS incubation is not very informative at the dose of bone marrow you use because the dose of about 2×10^8 bone marrow cells/kg is in the dog an about 10fold overdose. You can loose 9 tenths of your nucleated stem cells by your ALS incubation.
Kolb: We have shown in the CFU-C incubation with the unabsorbed ATG that you get complete CFU-C inhibition. I do not imply that the absorbed ATG is free of any toxicity of stem cells but with a high concentration (1:25) of the absorbed ATG we still see complete hemopoietic recovery in autologous bone marrow transplantation. It is very laborious to do dose experiments in dogs.
Kersey: Just a point for clarification related to your first set of experiments with the ATG-treated marrow: those dogs did not receive methotrexate post-grafting?
Kolb: No, they did not, nor any other immunosuppression.
Fliedner: Concerning the treatment of the gastrointestinal syndrome, can you tell us how you treat these dogs?
Kolb: The usual supportive care is that we look at these dogs twice a day and treat them with parental fluids, 300–400 ml twice a day, from the last irradiation until day 5. First they get boiled water and they get food from day six. We looked for a rise of temperature and they are treated with antibiotics.
Storb: I think that there is also a real GI syndrome in these dogs. We have essentially the same results as Dr. Kolb in both the single and fractionated irradiation. The GI syndrome occurs despite gentamycin and other antibiotics and careful adequate treatment. The dogs do not necessarily die of infections, they die in fact of real GI syndrome with protein deficiency and protein loss into the GI tract.

3 Immunocompetence and Histocompatibility in Bone Marrow Transplantation

Immunobiology of Minor Histocompatibility Antigens in the Lethal Graft-versus-Host-Reaction Induced in Adult Mice

Olga Halle-Pannenko, Linda L. Pritchard, and G. Mathé

A. Introduction

Although considerable progress has been made in the field of clinical bone marrow transplantation in recent years, the development of graft-versus-host (GVH) disease, and the rejection of the bone marrow after grafting between donor and recipient matched at the HL-A complex, are still relatively frequent phenomena. These observations strongly suggest that minor histocompatibility antigens (MiHA), or histocompatibility antigens coded for by genes located outside of the major histocompatibility complex (MHC), are important in determining the outcome of bone marrow transplants in man (Storb et al., 1977; van Rood et al., 1978). That non-MHC histocompatibility determinants are operative in affecting the outcome of bone marrow transplants was also suggested by results obtained in experiments using adult dogs (Storb et al., 1973; Rapaport et al., 1978) and mice (Cosgrove and Davies 1971; Rodey et al., 1974; Korngold and Sprent, 1978). However, while there is an increasing amount of evidence from clinical and experimental studies which suggests that minor histocompatibility antigens play an important role in organ transplantation, relatively little is known about the immunobiology of minor antigens, especially insofar as their role in the development of a lethal GVH reaction is concerned. Indeed, while the immunogenetics and immunobiology of such minor transplantation antigens have been rigorously studied using the host-versus-skin graft test in vivo (Graff, 1966; Wettstein and Frelinger, 1977[a]) and for some minor antigens, the cell-mediated lympholysis test in vitro (Bevan, 1976; Gordon and Simpson, 1977; Wettstein et al., 1977[b]), equivalent studies of systemic GVH disease have not been reported. This omission is an important one since neither immune reactions detectable by the available in vitro tests, nor host-versus-skin graft reactions, are exact equivalents of the GVH reaction. We know of no reports on MiHA-related GVH mortality induced in congenic strains of mice other than those reported by Cantrell and Hildemann (1972; 1973) using neonatal recipients; and as far as MiHA-related lethal GVH in adult man, dogs or mice is concerned, the absence of congenic strains in the first two species, and the fact that in mouse experiments reported to date the animals employed were unrelated strains serologically typed as H-2 identical, prevent one from asserting definitively that the transplantation reactions seen in adult recipients of bone marrow were developed against the MiHA incompatibility alone. This reservation is an important one, since disparities for mutations involving single regions of the H-2 complex can cause severe GVH disease (Klein, 1976), suggesting that small variations in the MHC may be highly immunogenic. Hence it is entirely plausible that serologically

undetected differences at the H-2 locus might contribute to the GVH mortality observed in non-congenic donor-recipient strain combinations. This type of reasoning may partially explain why, despite several reports suggesting the contrary, the antigens controlled by the major histocompatibility complex are often considered as "all-important" for development of lethal GVH disease, while the effects of MiHA are often considered as relatively insignificant in adult recipients; and the idea persists that, unless neonatal hosts or immunized donor cells are used, only a small proportion of the recipients eventually succumb to MiHA-induced GVH.

In the present work, using appropriate genetic systems (B10 congenic strains and various inbred strains of mice) we tried to answer several questions concerning the role of MiHA in lethal GVH developed in adult recipients of either normal or preimmunized donor cells. We have studied successively: a) primary GVH response to MiHA alone; b) primary response to MiHA associated with an additional incompatibility for H-2 antigens; c) secondary GVH response to MiHA; d) treatments capable of diminishing the MiHA-related lethal GVH reaction.

B. Material and Methods

1. *Animals:* Adult DBA/2 (D2; H-2^d), C57B1/10 (B10; H-2^b), (D2×B10.D2)F1 (H-$2^{d/d}$), (D2×B10.BR)F1 (H-$2^{d/k}$), (D2×B10)F1 (H-$2^{d/b}$), (Balb/c×B10)F1 (H-$2^{d/b}$), (B10.BR×B10.D2) F1 (H-$2^{k/d}$) and (B10×B10.D2)F1 (H-$2^{b/d}$) mice were purchased from Orléans La Source, CNRS Laboratories, France. Adult D2.GD (H-2 recombinant: K^d, I-A^d, I-B^b, I-J^b, I-E^b, I-C^b, ..., S^b, G^b, D^b) mice were kindly provided by Professeur J.-P. Levy, Hôpital Cochin, Paris, France.

2. *Induction of GVH:* Depending upon the experimental group, the recipients were (D2×B10.D2)F1, (D2×B10.BR)F1, (D2×B10)F1, (Balb/c×B10)F1, (B10.BR×B10.D2)F1 or (B10×B10.D2)F1 mice. In most cases, B10.D2 mice were used as donors, except in one series of experiments (§ C.II.1) where the donors were B10 mice. The F1 recipients were irradiated in toto at various doses (300, 500 and 1100 rads) using a cesium source (Rx 30/55M irradiator, Gravatom Industries Ltd., Gosport, Hampshire, England) at a dose rate of 106 rads/min. Within four hours after irradiation, mice of selected groups received, by intravenous injection, grafts of lymphoid or lymphoid and marrow cells; details of the experimental protocols are given in the results. All cell suspensions were prepared in RPMI 1640 medium containing glutamine (GIBCO Bio-Cult, Glasgow, Scotland), supplemented with kanamycin (50 μg/ml; Theraplix, Paris, France) and penicillin (100 IU/ml; Spécilline G, Specia, Paris). Viable cell counts were determined by the trypan blue dye exclusion method, and all cell doses refer to numbers of viable cells injected.

3. *Immunization of Donor Cells* was carried out according to the method described by Cerottini et al. (1971). Briefly, mice of various immunizing strains were lethally irradiated and reconstituted by intravenous injection with 5×10^7–10^8 donor spleen cells to be immunized; 5 days later, the spleens from such reconstituted mice contain approximately 90% donor-type cells.

C. Results

I. Lethal Graft-versus-Host (GVH) Reaction in Adult Recipients after Grafting of Normal Donor Cells Incompatible for Minor Histocompatibility Antigens (MiHA) Alone

In this series of experiments (D2 × B10.D2)F1 (H-$D^{d/d}$) mice were grafted with cells from the congenic resistant B10.D2 (H-2^d) strain, whose H-2^d allele is presumably identical to that of the D2 strain from which it is derived. Hence the GVH reaction which develops in this strain combination is clearly directed solely against D2 MiHA present in the recipient.

1. Augmentation of Susceptibility to MiHA-related Lethal GVH Reaction by Increasing the Irradiation Dose Used to Prepare Recipients for Grafting

The (D2 × B10.D2)F1 recipients were irradiated four hours before grafting at various doses, and then grafted with different types of cell suspensions from parent-strain B10.D2 donors. The results showed (Table 1) that in F1 recipients irradiated at 300 or 500 rads, no lethal GVH could be observed, regardless of the cell suspension grafted. In contrast, when F1 recipients were irradiated at 1100 rads, the grafting of 10^7 bone marrow and 8×10^6 spleen cells resulted in death of 100% of the recipients, while F1 recipients grafted with isogeneic cells survived indefinitely.

Table 1. Augmentation of susceptibility to MiHA-related lethal GVH reactions by increasing the irradiation dose used to prepare the recipients for grafting[a]

Donor Cells	Irradiation Dose (in rads)	Mortality: Median survival in days (range of mortality)
10^7 spleen	300	none
	500	
10^7 spleen + 2×10^7 lymph nodes	300	none
	500	
2×10^6 thymus + 8×10^6 spleen + 2×10^6 lymph nodes + 1.5×10^6 bone marrow	300	none
	500	
10^7 bone marrow + 8×10^6 spleen	300	none
	500	
10^7 bone marrow + 8×10^6 spleen	1,100	35 (20–51)
10^7 bone marrow + 8×10^6 spleen (isogeneic cell graft controls)	1,100	none
None (irradiation control)	1,100	12 (10–15)

[a] Marrow and/or lymphoid cells from normal B10.D2 donors were grafted (i.v.) into adult (D2 × B10.D2)F1 hybrid mice, irradiated at the doses indicated

2. MiHA-Related Lethal GVH Reaction Requires the Presence of Small Numbers of Mature Immunocompetent Cells in the Marrow-Cell Inoculum

In order to study the possible requirement for mature immunocompetent cells for the development of MiHA-related GVH, various numbers of spleen cells were added to the bone marrow inoculum. As shown in Table 2, the severity of the GVH reaction was correlated with the number of spleen cells added; no mortality was observed after grafting of bone marrow cells alone, although the presence of as few as 10^6 additional spleen cells permitted the induction of mortality in 57% of grafted recipients.

Table 2. MiHA-related lethal GVH reactions require the presence of small numbers of mature immunocompetent cells in marrow cell inoculum[a]

Donor cells	Mortality: Median survival in days (range of mortality)
None (irradiation control)	12 (10–15)
10^7 bone marrow + 8×10^6 spleen	38 (10–60)
10^7 bone marrow + 4×10^6 spleen	38 (32–83)
10^7 bone marrow + 2×10^6 spleen	79 (36–>120)
10^7 bone marrow + 10^6 spleen	103 (54–>120)
10^7 bone marrow	none

[a] Marrow and spleen cells from normal B10.D2 donors were grafted (i.v.) into adult (D2 × B10.D2)F1 hybrid mice irradiated at 1100 rads

In all experiments, graft recipients were subjected to microbiological and histopathological testing in order to verify that death was due neither to infection nor to aplasia. Results of these tests indicated that the mortality observed was, in fact, a consequence of GVH disease. Moreover, the GVH reaction directed against D2 MiHA in this system is accompanied by a special type of "secondary disease": a macroscopically detectable paniculitis with edema and characteristic histopathological changes (Rappaport et al., 1979).

II. Lethal GVH Reaction in Adult Recipients After Grafting of Donor Cells Incompatible for MiHA and H-2 Antigens

In order to study the combined effects of MiHA and H-2 antigens, donor-recipient strain combinations incompatible for either MiHA alone, or H-2 antigens alone, or for both MiHA and H-2 antigens were used in this series of experiments.

1. For a Given H-2 Haplotype the Effects of MiHA and H-2 May or May Not Be Cumulative, Depending on the Nature of the MiHA

GVH mortality was compared after grafting of parent-strain (B10) donor cells ($H\text{-}2^b$) to F1 recipients ($H\text{-}2^{b/d}$) incompatible for either $H\text{-}2^d$ antigens alone or for $H\text{-}2^d$ antigens plus MiHA of two different strains (D2 or Balb/c). The results showed (Fig. 1) that the mortality induced by incompatibility for $H\text{-}2^d$ plus D2-strain MiHA is significantly more rapid than mortality induced by incompatibility for $H\text{-}2^d$ antigens alone; this indicates that the effects of $H\text{-}2^d$ antigens and D2 MiHA are cumulative; in contrast, the mortality induced by incompatibility for $H\text{-}2^d$ plus Balb/c MiHA does not differ significantly from the mortality induced by incompatibility for $H\text{-}2^d$ antigens alone, indicating that the effects of $H\text{-}2^d$ and Balb/c MiHA are not cumulative. Thus, for a given H-2 haplotype, a cumulative effect of an additional incompatibility for MiHA may be observed or may not be observed, depending on the nature of the MiHA.

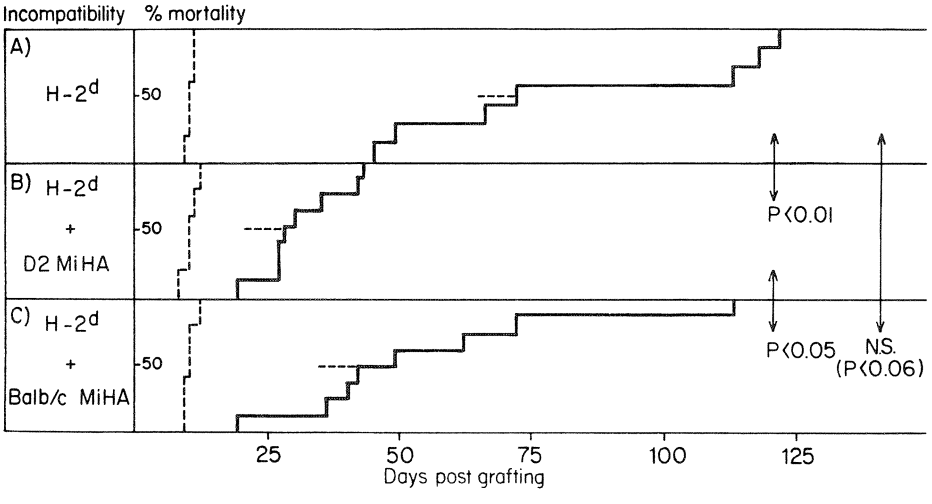


Fig. 1. For a given H-2 haplotype the effects of MiHA and H-2 antigens may or may not be cumulative, depending on the nature of the MiHA. In all groups, 10^7 bone marrow cells from normal B10 donors were grafted (i.v.) into adult (B10×B10.D2)F1 *(A);* (B10×D2)F1 *(B)* and (B10×Balb/c)F1 *(C)* hybrid mice, irradiated at 1100 rads. Irradiation control: – – –; grafted mice: ——. Statistics: Wilcoxon's rank-sum non-parametric test

2. For a Given Set of MiHA the Effects of MiHA and H-2 Antigens May Be Cumulative or "Suppressive", Depending on the H-2 Haplotype

In these experiments we have studied whether the effect of the same (D2) MiHA can vary as a function of the H-2 haplotype with which they are associated; we have compared the mortality induced after grafting of the same parent-strain (B10.D2) donor cells to various F1 recipients incompatible for: D2 MiHA alone; H-2 (k or b) antigens alone; or D2 MiHA plus H-2 (k or b) antigens.

a) Effects of D2 MiHA and H-2^k Antigens Are Cumulative

As shown in Fig. 2, incompatibility for D2 MiHA alone led to less rapid onset of mortality than did incompatibility for H-2^k alone or H-2^k plus D2 MiHA. On the other hand, when compared with mortality induced by incompatibility for H-2^k alone, an additional incompatibility for MiHA accelerated the mortality; these results indicate that the effect of H-2^k antigens and D2 MiHA are cumulative.

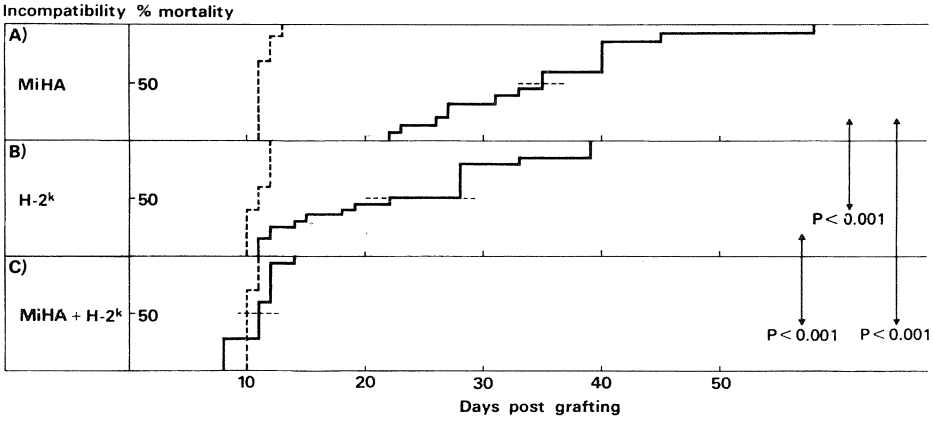


Fig. 2. Effects of D2 MiHA and H-2^k antigens are cumulative. In all groups, 10^7 bone marrow and 8×10^6 spleen cells from normal B10.D2 donors were grafted (i.v.) into adult (D2 × B10.D2)F1 *(A)*; (B10.BR × B10.D2)F1 *(B)* and (D2 × B10.BR)F1 *(C)* hybrid mice irradiated at 1100 rads. Irradiation control: – – – –; grafted mice: ———. Statistics: Wilcoxon's rank-sum non-parametric test

b) Effects of D2 MiHA and H-2^b Antigens Are "Suppressive"

In the same experiment, we also studied the effect of an additional incompatibility for D2 MiHA associated with incompatibility for H-2^b antigens. The results showed (Fig. 3) that incompatibility for D2 MiHA alone led to less rapid mortality than did incompatibility for H-2^b alone. Surprisingly, when compared with the mortality induced by incompatibility for H-2^b alone, an additional incompatibility for D2 MiHA *delayed* the mortality. Figure 4 shows the results obtained in five independent experiments, where in all cases the mortality induced by incompatibility for D2 MiHA plus H-2^b was less rapid than that induced by incompatibility for H-2^b antigens alone, indicating that the effects of an additional incompatibility for D2 MiHA antigens was not cumulative but that, in contrast, it *decreased* the anti-H-2^b response. Hence, for a given set of MiHA the effects of MiHA and H-2 antigens may be cumulative or "suppressive" depending on the H-2 haplotype with which the MiHA are associated. It can be pointed out that, interestingly, the cumulative effect of MiHA and H-2 antigens was found in the situation where the response to H-2 antigens alone was relatively weak (H-2^k; median survival time (MST) = 22 days; range of mortality 11 to 39 days) while it was not found when the response to H-2 antigens alone was relatively strong (H-2^b; MST = 15 days; range of mortality 9 to 24 days), as compared to the response to MiHA alone (MST = 35 days; range of mortality 22 to 60 days). This observation will be discussed later.

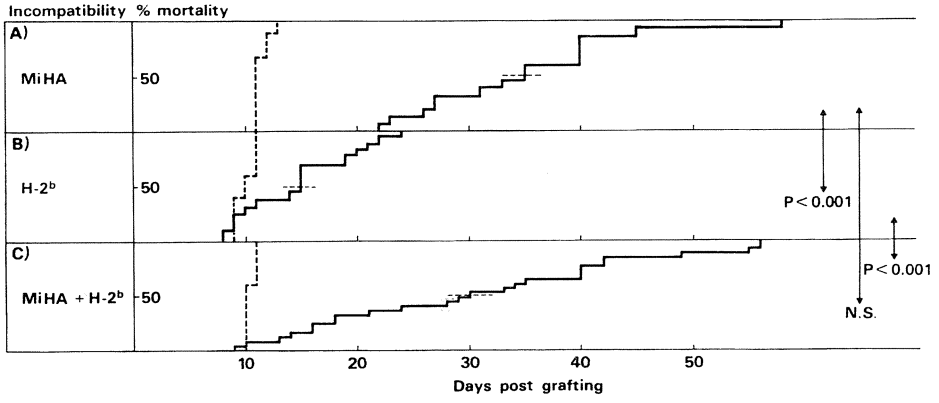


Fig. 3. Effects of D2 MiHA and H-2^b antigens are "suppressive". In all groups, 10^7 bone marrow and 8×10^6 spleen cells from normal B10.D2 donors were grafted (i.v.) into adult (D2 × B10.D2)F1 *(A);* (B10 × B10.D2)F1 *(B)* and (D2 × B10)F1 *(C)* hybrid mice irradiated at 1100 rads. Irradiation control: – – – –; grafted mice: ———. Statistics: Wilcoxon's rank-sum non-parametric test

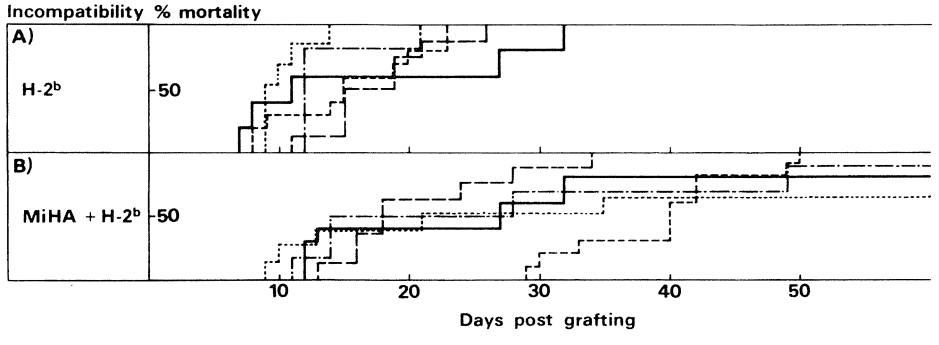


Fig. 4. "Suppressive" effect of D2 MiHA and H-2^b antigens. Results of five independent experiments. In all groups, 10^7 bone marrow and 8×10^6 spleen cells from normal B10.D2 donors were grafted (i.v.) into adult (B10 × B10.D2)F1 *(A)* and (D2 × B10)F1 *(B)* hybrid mice irradiated at 1100 rads

III. Secondary Response to MiHA

1. Secondary Response to MiHA Is Suppressed After Donor Immunization Against MiHA and H-2^k or H-2^b Antigens

In these experiments we have studied the lethal GVH reaction after grafting of immunized donor cells. In all experimental groups the donors and recipients were incompatible for D2 MiHA alone, but the donor cells were preimmunized against strains incompatible for a) D2 MiHA alone; b) D2 MiHA and H-2^k antigens and c) D2 MiHA and H-2^b antigens. The results showed that preimmunization of the donor against D2 MiHA alone led to earlier onset of D2-MiHA-related mortality as compared to that caused by non-immunized donor cells; however, this "secondary response"-type mortality was significantly delayed when donor cells were immunized simultaneously against D2 MiHA and H-2^k antigens (Fig. 5) or

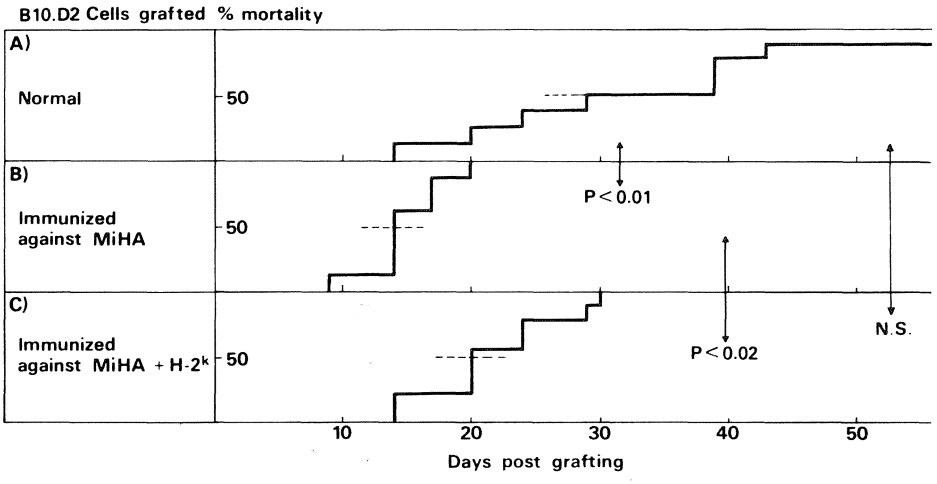


Fig. 5. Secondary response to MiHA is suppressed after donor immunization against MiHA and H-2^k antigens. In all groups, 10^7 bone marrow and 8×10^6 spleen cells from B10.D2 donors were grafted (i.v.) into adult (D2×B10.D2)F1 hybrid mice irradiated at 1100 rads. Spleen cells were either normal *(A)* or immunized against (D2×B10.D2)F1 *(B)* or (D2×B10.BR)F1 *(C)* strains. Statistics: Wilcoxon's rank-sum non-parametric test

against D2 MiHA and H-2^b antigens (Fig. 6), resulting in a mortality pattern comparable to that induced by non-immunized donor cells. Therefore, if donors are primed simultaneously with MiHA and H-2 antigens, the secondary response to MiHA alone may be completely suppressed.

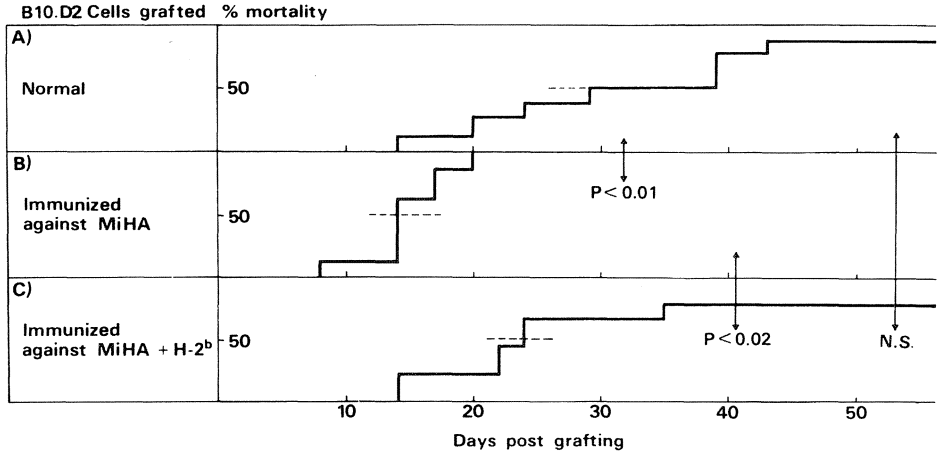


Fig. 6. Secondary response to MiHA is suppressed after donor immunization against MiHA and H-2^b antigens. In all groups, 10^7 bone marrow and 8×10^6 spleen cells from B10.D2 donors were grafted (i.v.) into adult (D2×B10.D2)F1 hybrid mice irradiated at 1100 rads. Spleen cells were either normal *(A)* or immunized against (D2×B10.D2)F1 *(B)* or (D2×B10)F1 *(C)* strains. Statistics: Wilcoxon's rank-sum non-parametric test

2. Suppression of Secondary Response to MiHA After Donor Immunization Against MiHA and H-2^b Antigens Is Caused by Incompatibility at K and/or I-A Regions of the H-2 Complex

In one experimental group of this series of experiments, donor cells were preimmunized against an H-2 recombinant strain (D2.GD) incompatible for D2 MiHA and H-2^b antigens coded for by the I-B → D regions, but compatible (H-2^d) for the antigens coded for by the K and I-A regions of the H-2 complex. As shown in Fig. 7, the mortality induced in this group was identical to that induced by donor immunization against MiHA alone, present in either (D2 × B10.D2)F1 hybrid or D2 inbred mice. Hence, the secondary response to MiHA was not suppressed in the absence of incompatibility for H-2^b antigens coded for by K and I-A regions, suggesting that the suppressive effect of simultaneous priming against H-2 antigens is caused by K and/or I-A region incompatibilities.

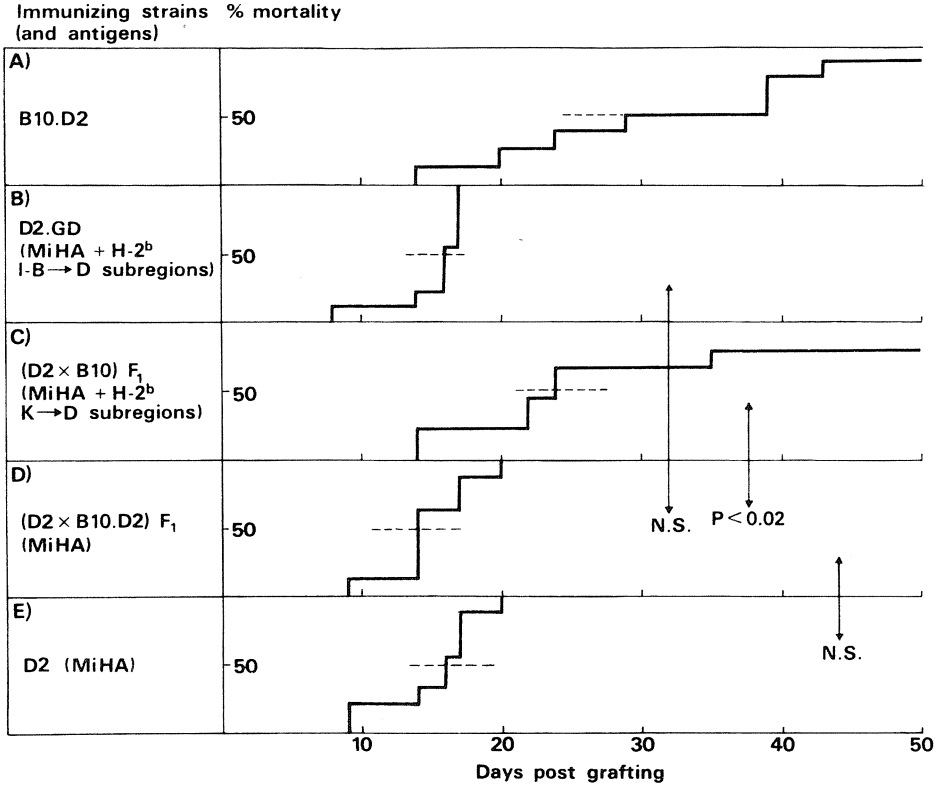


Fig. 7. Suppression of secondary response to MiHA after donor immunization against MiHA and H-2^b antigens is caused by incompatibility for K and/or I-A regions of the H-2 complex. In all groups, 10^7 bone marrow and 8×10^6 spleen cells from B10.D2 donors were grafted (i.v.) into adult (D2 × B10.D2)F1 hybrid mice irradiated at 1100 rads. Spleen cells were either normal *(A)* or immunized against: D2 MiHA plus H-2^b antigens controlled by the I-B → D regions (in the absence of incompatibility for antigens coded for by K and I-A regions *(B)*; D2 MiHA and all H-2^b antigens *(C)*; D2 MiHA alone (heterozygous F1 strain) *(D)*; D2 MiHA alone (homozygous parent-strain) *(E)*. Statistics: Wilcoxon's rank-sum non-parametric test

3. Immunization of the Donor Against MiHA and H-2^b or H-2^k Antigens Induces Suppressor Cells Capable of Diminishing the MiHA-Related Lethal GVH Reaction

The results described above, indicating that the secondary response to MiHA may be suppressed by donor preimmunization against MiHA and H-2 antigens, led us to study whether this phenomenon was related to the induction of suppressor cells. In all groups of this series of experiments donor and recipient were incompatible for MiHA alone. In order to search for the presence of suppressor cells postulated to be induced by immunization against MiHA and H-2 antigens, and to test their effect on MiHA-related GVH, normal marrow and spleen cells from B10.D2 donors were supplemented with B10.D2 spleen cells preimmunized in lethally irradiated "primary recipients" incompatible for D2 MiHA and H-2^k or H-2^b antigens. Before addition to normal cells, the immunized cells were irradiated at 1200 rads. The mortality induced by normal cells supplemented with immunized cells was compared to that induced by normal cells alone. As shown in Fig. 8 (experiment 1), the addition of cells immunized against MiHA and H-2^k or H-2^b delayed the mortality as compared to that induced by the normal cells alone. However, it should be pointed out that, in this experiment, the mortality induced by normal cells was significantly more rapid than is usually the case; and one might be tempted to ask whether this suppressive effect can be observed when the mortality induced by normal cells is less rapid. The results obtained in experiment 2 of Fig. 8 indicate that this is indeed the case; in this experiment, we compared the mortality induced by normal cells alone with that induced by normal cells supplemented with cells immunized against D2 MiHA plus H-2^k antigens, and to that induced in an additional control group where normal cells were supplemented with cells which had been "activated" in lethally irradiated isogeneic "primary recipients". The mortality induced by a mixture of normal cells and irradiated "activated" cells was, surprisingly, significantly more rapid than that induced by normal cells alone (Fig. 8; experiment 2). This indicates that the 1200 rad dose did not abolish the GVH potential. In spite of this fact, when the additional irradiated cells were preimmunized against D2 MiHA and H-2^k, the mortality was significantly delayed as compared with that induced by either a mixture of normal plus activated cells or normal cells alone. Consequently, an "infectious" effect of the preimmunized cells was clearly shown, indicating that immunization against MiHA and H-2 antigens induces radioresistant suppressor cells capable of diminishing the anti-MiHA GVH response.

IV. Consequences for the Control of the MiHA-Related Lethal GVH Reaction

It is now quite clear that MiHA can play an important role in GVH-reaction-induced mortality in both H-2 compatible and H-2 incompatible donor-recipient combinations. Hence means for controlling the GVH reaction must be developed, since perfect donor-recipient matching for all histocompatibility antigens is virtually unattainable (except in the case of identical twins, triplets, etc....).

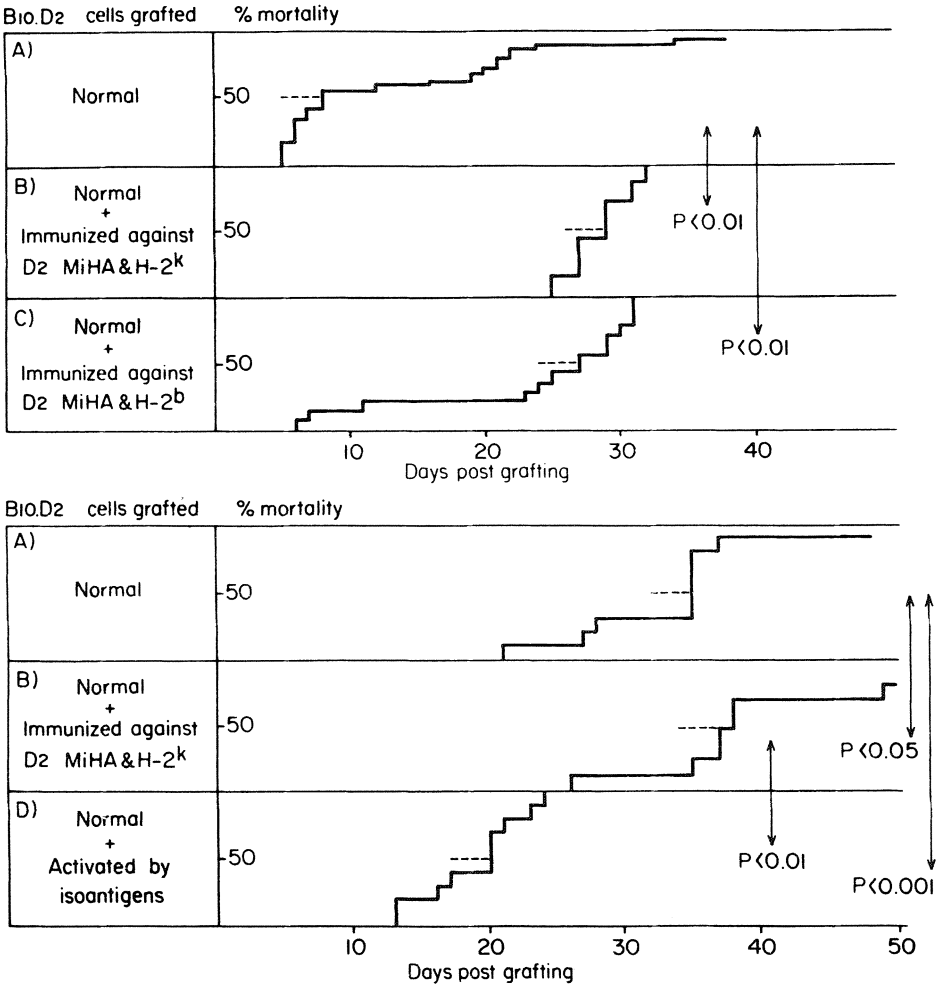


Fig. 8. Immunization of the donors against MiHA and H-2^b or H-2^k antigens induces suppressor cells capable of diminishing the MiHA-related lethal GVH reaction. In all groups, 10^7 bone marrow and 8×10^6 spleen cells from normal B10.D2 donors were grafted (i.v.) into adult (D2 × B10.D2)F1 hybrid mice irradiated at 1100 rads. These normal donor cells were grafted either alone *(A)* or along with 8×10^6 irradiated (1200 rads in vitro) spleen cells immunized against (D2 × B10.BR)F1 *(B)* or (D2 × B10)F1 *(C)* strains; or activated in isogeneic B10.D2 recipients *(D)*. Statistics: Wilcoxon's rank-sum non-parametric test

We showed previously that lipoprotein preparations of H-2 antigens extracted from liver can abrogate secondary disease due to a GVH reaction induced across a *major* histocompatibility barrier (Mathé et al., 1979). On the other hand, the results described in the present paper, indicating that the response to MiHA may be decreased after donor immunization against MiHA and H-2 antigens (but not after donor immunization against MiHA alone), suggest that, for the induction of suppression of the anti-MiHA response, an immune reaction against foreign H-2 antigens is sufficient. All these results taken together led us to

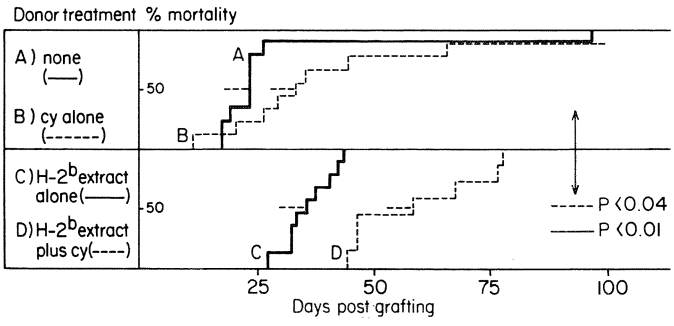


Fig. 9. Delay in MiHA-related lethal GVH by donor pretreatment with an extract containing unrelated H-2 antigens. Recipients were adult (D2×B10.D2)F1 mice (H-2^b) irradiated at 1100 rads and grafted i.v. with 10^7 bone marrow and 8×10^6 spleen cells from normal or pretreated B10.D2 donors (H-2^d). H-2 antigens (lipoprotein extract) were administered at the dose of 5 mg/donor mouse on days –13 and –10; lipoprotein extract was prepared from livers of B10 mice (H-2^b) as previously described (Halle-Pannenko et al., 1971). Cyclophosphamide (Cy) was injected (200 mg/kg; i.p.) on day –7. Statistics: Wilcoxon's rank-sum non-parametric test

ask whether the GVH reaction directed against MiHA alone could be modified by donor treatment with a lipoprotein preparation of H-2 antigens extracted from the liver of an unrelated strain. Results showed (Fig. 9) that the pretreatment of B10.D2 donors (H-2^d; B10 MiHA) with lipoprotein liver-extract from B10 strain, different for H-2^b antigens alone, (H-2^b; B10 MiHA), significantly delayed MiHA-related GVH-mortality in (D2×B10.D2)F1 recipients (H-2^d; B10 and D2 MiHA). In addition, donor pretreatment with B10 extract in conjunction with cyclophosphamide (Cy) delayed mortality even more. These results indicate that, in a system where the GVH reaction is directed solely against D2 MiHA in an H-2^d context, mortality is delayed by donor treatment with an unrelated (H-2^b) extract, and that this alloimmunization-induced suppression is potentiated by an additional treatment with Cy.

D. Discussion

The results described in the present report clearly show that incompatibility for MiHA alone may induce a high rate of GVH mortality in adult (D2×B10.D2)F1 recipients of normal bone marrow cells from B10.D2 donors; however, for this mortality to be induced, the presence of mature immunocompetent cells in marrow graft is required. High dose irradiation of the recipients considerably increases their sensitivity to MiHA-related lethal GVH. Since a parent-to-F1 donor-recipient combination was employed, it is unlikely that this phenomenon is related to a decreased host-versus-graft reaction subsequent to radiation-induced immunosuppression. Mice dying in this system from MiHA-related GVH show particular histopathological changes; and in about 70% of them a macroscopically detectable paniculitis with edema develops (Halle-Pannenko et al., 1979; Rappaport et al., 1979).

While the effects of incompatibility involving several MiHA plus H-2 antigens have been extensively studied in the skin graft model and have been

found to be cumulative (Graff, 1966) there is little if any information on the combined effects of MiHA and H-2 antigens in the GVH reaction. Our results showed that the effects of MiHA and H-2 may, in some cases, be cumulative, but that these effects vary depending on a) the nature of the MiHA involved as well as on b) the H-2 haplotype with which a given set of MiHA are associated; and that, in some genetic combinations, the response to MiHA plus H-2 antigens, instead of being stronger, may be weaker than the response to H-2 antigens alone. Thus, in the lethal GVH reaction induced by grafting the same H-2^b donor cells, the effects of H-2^d antigens and D2 MiHA were cumulative, while no cumulative effect was found for H-2^d antigens and Balb/c MiHA; this indicates that the combined effects of MiHA and H-2 antigens may be cumulative, and that, for a given H-2 haplotype, a cumulative effect may or may not be detected, depending on the nature of these MiHA. On the other hand, when we studied the effect of D2 MiHA associated with different H-2 haplotypes, the results showed that after grafting of the same H-2^d donor cells, the response to D2 MiHA and H-2^k antigens was *stronger* than the response to H-2^k alone, while the response to D2 MiHA and H-2^b antigens was, surprisingly, *weaker* than the response to H-2^b antigens alone. Thus, the effect of an additional incompatibility for a given set of MiHA may be cumulative or suppressive, depending on the H-2 haplotype with which these MiHA are associated. It is interesting to note that a cumulative effect of MiHA and H-2 antigens was found in the situation where the response to H-2 antigens alone was relatively weak (case of the response to H-2^k) while it was not found in the situation where the response to H-2 antigens alone was relatively strong (case of the response to H-2^b) as compared to the response to MiHA alone. This result supports the hypothesis of "independent effects" proposed by Bailey (1971) as an explanation for the cumulative effect of histocompatibility antigens. This hypothesis of "independent effects" predicts that the shift to the left will be greater the closer the MST's of the two antigenic differences are to coinciding, and the greater is the overlapping interval in the distributions of survival times. The results obtained by Graff (1966) with skin graft survivals are in agreement with this prediction. In our experiments, the MST induced by MiHA (35 days) is closer to the MST induced by H-2^k (22 days) than it is to the MST induced by H-2^b (15 days) antigens. Moreover, the distribution of mortality induced by MiHA alone overlaps with that induced by H-2^k alone during 17 days, and with that induced by H-2^b antigens alone during only 4 days. However, while the hypothesis of "independent effects" may explain why a cumulative effect is found for D2 MiHA and H-2^k antigens but not for D2 MiHA and H-2^b antigens, it does not explain why the effect of D2 MiHA and H-2^b antigens is not equal to but is, rather, weaker than the effect of H-2^b antigens alone. We have not established what mechanism is involved in this phenomenon, but the induction of suppressor cells capable of neutralizing the concomittant killing (cytotoxic?) process could provide an explanation. It may be envisaged that MiHA and H-2 antigens induce cytotoxic cells and suppressor cells simultaneously, and that they do so in the case where the MiHA are associated with H-2^b as well as when they are associated with H-2^k antigens. In the presence of the H-2 target, the effect of suppressor cells is detectable (in the case of H-2^b) or is not detectable (in the case of H-2^k), depending on the H-2 haplotype and the intensity and/or kinetics of the

concomittant anti-H-2 cytotoxic response. This hypothesis is corroborated by a further result (discussed below) indicating that donor preimmunization against MiHA plus H-2^b or H-2^k induces suppressor cells whose effects are detectable in the *absence* of the H-2 target, i.e. when tested in recipients incompatible for MiHA alone.

Indeed, in this series of experiments the preimmunization of donors of H-2^d cells resulted in earlier onset of MiHA-related mortality as compared to that caused by non-immunized cells; immunization against the MiHA-homozygous parent-strain (D2) yielded the same results as did immunization against a MiHA-heterozygous F1 strain, suggesting that under these experimental conditions, the "gene dose" effect does not significantly influence the "secondary-type" GVH response. However, this "secondary-response"-type mortality is completely suppressed if the H-2^d donors are primed simultaneously with MiHA and H-2^k or H-2^b antigens and then tested in the recipients differing by MiHA alone. The suppressive effect was not found when H-2^d donors were preimmunized against H-2^b antigens coded by I-B→D regions, in the absence of incompatibility for the antigens coded by the K and I-A regions of the H-2 complex, suggesting that the suppressive effect is caused by K and/or I-A region incompatibilities.

These results led us to verify whether the present suppression is due to the induction of suppressor cells; the results clearly demonstrated the "infectious" effect of the cells preimmunized against MiHA and H-2^k or H-2^b antigens, and indicated that these suppressor cells are capable of diminishing the GVH mortality caused by incompatibility for MiHA alone. They also indicated that the suppressor cells are resistant to irradiation at 1200 rads. We are currently studying the nature of these suppressor cells.

Since perfect donor-recipient matching is virtually unattainable, given the great number of histocompatibility antigens which exists, means for controlling the GVH reaction should be developed. We reported previously that donor pretreatment with lipoprotein preparations containing H-2 antigens extracted from liver (Mathé et al., 1979) can be used prophylactically to abrogate lethal GVH reactions developed across an H-2 barrier. The fact that, in the present experiments, donor immunization against MiHA plus H-2 antigens (but not against MiHA alone) delayed the lethal GVH reaction developed across a MiHA barrier, tempted us to speculate that, in donor-recipient combinations differing for MiHA alone, donor pretreatment with a lipoprotein preparation containing H-2 antigens extracted from an "unrelated" strain (with a different H-2 haplotype) might be sufficient to decrease the severity of the anti-MiHA GVH reaction. Results indicate that this is indeed the case. In a genetic system where the GVH reaction was directed solely against D2 MiHA in an H-2^d context, the mortality was delayed by donor treatment with an unrelated (H-2^b) extract; and donor pretreatment with this same extract followed by single high dose (200 mg/kg) of cyclophosphamide delayed mortality even more. Using the same genetic system we have also observed (Halle-Pannenko et al., 1979) that donor pretreatment with B10 extract in conjunction with a D2 extract delayed mortality more than did the B10 extract alone.

We are currently studying the mechanism and specificity of these effects. As far as the suppression by H-2 antigens alone is concerned, it may be noticed that

the lipoprotein preparation used was extracted from liver, and that, consequently, it is presumed not to contain Ia (I-region-coded) antigens; taken together with results obtained after donor immunization against the D2.GD strain (which indicated that the suppression of the anti-MiHA response by H-2^b is related to incompatibility for K and/or I-A regions), these results tend to imply that the suppression of the anti-MiHA response by H-2^b is related to K-region incompatibility.

In conclusion: MiHA can play an important role in the lethal GVH reaction induced in both MHC-matched and MHC-mismatched donor-recipient combinations. The combined effects of MiHA and H-2 antigens are complex and vary as a function of a) the nature of the MiHA, as well as b) the H-2 haplotype. As the response to MiHA may both decrease and be decreased by a concomittant anti-H-2 response, it is strongly suggested that the expression and/or recognition of MiHA and H-2 antigens is inter-dependent. Since perfect donor-recipient matching for all MiHA is unattainable for theoretical reasons, dependable means for controlling the GVH reaction must be developed; on the basis of the present results one is tempted to speculate that in donor-recipient combinations matched at the MHC, pretreatment of the *donor* with "third-party" antigens (for example by blood transfusions from an individual with a different MHC haplotype) might be sufficient to diminish the severity of the anti-MiHA GVH reaction, through the induction of suppressor cells. On the other hand, it is important that the GVH response to MiHA be subjected to the same intensive study, and characterized in as much detail as is the response to MHC-coded antigens; and the development of rapid, sensitive in vitro tests for MiHA should be given high priority.

E. Summary

The role of minor histocompatibility antigens (MiHA) in the lethal graft-versus-host (GVH) reaction was studied using B10 congenic strains and various inbred strains of adult mice, in experiments involving: 1. the primary GVH response to MiHA alone; 2. primary GVH response to MiHA associated with an additional incompatibility for H-2 antigens; 3. secondary GVH response to MiHA; 4. treatments capable of diminishing the MiHA-related GVH reaction. The results showed that: 1. disparity for DBA/2 (D2) MiHA alone is sufficient to induce severe lethal GVH disease in adult recipients of normal cells, but the presence of (relatively small numbers of) mature immunocompetent cells is required along with the marrow graft. High-dose irradiation of adult recipients increases their susceptibility to D2-MiHA-related GVH disease; 2. the effects of MiHA and H-2 antigens may in some cases be cumulative, but these effects vary, depending upon: a) the nature of the MiHA, as well as b) the H-2 haplotype with which they are associated; 3. preimmunization of the donors against D2 MiHA accelerates the D2-MiHA-related lethal GVH reaction; however this secondary-response type mortality is completely suppressed if the donors are primed simultaneously with D2 MiHA and H-2^k or H-2^b antigens and then tested in the recipients differing for D2 MiHA alone. Results obtained after preimmunization against an

H-2 recombinant strain (D2.GD) suggested that the suppression of the secondary response to D2 MiHA after donor immunization against D2 MiHA and H-2^b antigens is caused by incompatibility for the K and/or I-A regions of the H-2 complex. Immunization of the donor against D2 MiHA and H-2^b or H-2^k antigens induces suppressor cells capable of diminishing the D2-MiHA-related lethal GVH reaction. 4. Finally, in a genetic system where the GVH reaction was directed solely against D2 MiHA in an H-2^d context, mortality was delayed by donor treatment with an unrelated (H-2^b) lipoprotein extract; and donor pretreatment with this same extract followed by a single high dose (200 mg/kg) of cyclophosphamide delayed mortality even more. The lipoprotein preparation used was extracted from liver, and is consequently presumed not to contain Ia (I-region-coded) antigens; taken together with results obtained after donor immunization against the D2.GD strain, these results tend to imply that suppression of the anti-MiHA response by H-2^b is related to K-region incompatibility. It is concluded that MiHA can play an important role in the lethal GVH reaction induced in both H-2 compatible and H-2 incompatible donor-recipient combinations. The combined effects of MiHA and H-2 antigens are complex, and strongly suggest that the expression and/or recognition of MiHA and H-2 antigens is inter-dependent. On the basis of the present results, one is tempted to speculate that, in donor-recipient combinations matched at the major histocompatibility complex (MHC), pretreatment of the donor with a "third-party" (MHC-unrelated) blood transfusion might be sufficient to diminish the severity of the MiHA-related lethal GVH reaction.

Acknowledgements

The authors gratefully acknowledge the competent technical assistance of Ms. Laurence Fanouillaire, Mrs. Michelle Grégoire, and Mrs. Nicole Riché.

This work was supported by grants n° 76.7.0967 from the Délégation Générale à la Recherche Scientifique et Technique, and n° ATP 43.76.75 and ATP 59.78.91 from the Institut National de la Santé et de la Recherche Médicale.

References

Bailey, D. W.: Cumulative effect or independent effect? Transplantation *11*, 419–422 (1971)

Bevan, M. J.: H-2 restriction of cytolysis after immunization of minor H congenic pairs of mice. J. Immunogenet. *3*, 177–184 (1976)

Cantrell, J. L., Hildemann, W. H.: Characteristics of histocompatibility barriers in congenic mice. Transplant. Proc. *5*, 271–274 (1973)

Cerottini, J. C., Nordin, A. A., Brunner, K. T.: Cellular and humoral response to transplantation antigens. I. Development of alloantibody-forming cells and cytotoxicity lymphocytes in the graft-versus-host reaction. J. Exp. Med. *134*, 553–564 (1971)

Cosgrove, G. E., Davies, M. L.: The effect of presensitization of parental donors on graft-versus-host disease in irradiated F1 hybrid mice. Proc. Soc. Exp. Biol. Med. *138*, 210–212 (1971)

Gordon, R. D., Simpson, E.: Immune-response gene control of cytotoxic T cell response to H-Y. Transplant. Proc. *9*, 885–888 (1977)

Graff, R. J., Silvers, W. K., Billingham, R. E., Hildemann, W. H., Snell, G. D: The cumulative effect of histocompatibility antigens. Transplantation *4*, 605–617 (1966)

Halle-Pannenko, O., Martyre, M. C., Mathé, G.: Prevention of graft-versus-host reaction by donor pretreatment with soluble H-2 antigens. Transplantation *11,* 414–417 (1971)

Halle-Pannenko, O., Pritchard, L. L., Mathé, G.: Minor histocompatibility antigens and lethal graft-versus-host reaction in adult mice. In: Graft-versus-leukemia in man and animal models. Okunewick, J. P., Meredith, R. F. (eds.). CRC Press 1979

Klein, J., Chiang, C. L.: Ability of H-2 regions to induce graft-versus-host disease. J. Immunol. *117,* 736–740 (1976)

Korngold, R., Sprent, J.: Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. J. Exp. Med. *148,* 1687–1698 (1978)

Rapaport, F. T., Bachvaroff, R. J., Watanabe, K., Hirasawa, H., Mollen, N., Ferrebee, J. W., Amos, D. B., Cannon, F. D., Blumenstock, D. A.: Induction of allogeneic unresponsiveness in adult dogs. Role of non-DLA histocompatibility variables in conditioning the outcome of bone marrow, kidney and skin transplantation in radiation chimeras. J. Clin. Invest. *61,* 790–799 (1978)

Rappaport, H., Khalil, A. M., Halle-Pannenko, O., Pritchard, L. L., Dantchev, D., Mathé, G.: Histopathologic sequence of events in adult mice undergoing lethal graft-versus-host reaction developed across H-2 and/or non-H-2 histocompatibility barriers. Amer. J. Pathol. *96,* 121–142 (1979)

Rodey, G. E., Bortin, M. M., Bach, F. H., Rimm, A. A.: Mixed leukocyte culture reactivity and chronic graft-versus-host reactions (secondary disease) between allogeneic H-2^k mouse strains. Transplantation *17,* 84–88 (1974)

Rood, J. J. van, Leeuwen, A. van, Goulmy, E., Termijtelen, A., Bradley, B. A., Brand, A., Eernisse, J.: The importance of non-HLA systems and the feasibility of the use of unrelated donors in bone marrow transplantation. Transplant. Proc. *10,* 47–51 (1978)

Salaman, M. H., Wedderburn, N., Festenstein, H., Huber, B.: Detection of a graft-versus-host reaction between mice compatible at the H-2 locus. Transplantation, *16,* 29–31 (1973)

Storb, R., Rudolph, R. H., Kolb, H. J., Graham, T. C., Mickelson, E., Erickson, V., Lerner, K. G., Kolb, H., Thomas, E. D.: Marrow grafts between DL-A matched canine littermates. Transplantation, *15,* 92–100 (1973)

Storb, R., Weiden, P. L., Prentice, R., Buckner, C. D., Clift, R. A., Einstein, A. B., Fefer, A., Johnson, F. L., Lerner, K. G., Neiman, P. E., Sanders, J. E., Thomas, E. D.: Aplastic anemia (AA) treated by allogeneic marrow transplantation: The Seattle experience. Transplant. Proc. *9,* 181–185 (1977)

Wettstein, P. J., Haughton, G., Frelinger, J. A.: H-2 effects on cell-cell interactions in the response to single non-H-2 alloantigens. I. Donor H-2D region control of H-7.1 immunogenicity and lack of restriction in vivo. J. Exp. Med. *146,* 1346–1355 (1977a)

Wettstein, P. J., Frelinger, J. A.: H-2 effects on cell-cell interactions in the response to single non-H-2 alloantigens. II. H-2D region control of 7.1-specific stimulator function in mixed lymphocyte culture and susceptibility to lysis by H-7.1 specific cytotoxic cells. J. Exp. Med. *146,* 1356–1366 (1977b)

Discussion

Thierfelder: Concerning your suppressive effect on GVH I remember an early paper from your Institute where marrow donors were immunized against recipients and got a milder GVH. You interpreted this as an enhancement phenomenon. My question is, could you also interpret your phenomenon as enhancement or, in other words, could you exclude enhancement by using serum of the donor?

Halle-Pannenko: You speak of our work on GVH abrogation across H-2 barrier. There we found indeed a protective factor. We postulated an antigen-antibody complex, since we could not show by classical serological tests the presence of an antibody. As to the present model, we did not study its mechanism yet. Theoretically it cannot be excluded that what we postulated to be antigen-antibody complexes were perhaps a soluble suppressive factor.

The Role of MHC Gene Products in the Development of the T-Cell Repertoire

H. Waldmann, A. Munro and P. H. Maurer

A. Introduction

The thymus is a necessary differentiation organ for the proper acquisition of T-lymphocyte competence. In any evaluation of the merits of bone marrow transplantation between HLA mismatched (partial or total) combinations it is necessary to acquire data on the functional status of newly developing donor T-cells which have matured to competence in the recipient thymus. The basis for this concern comes in large part from studies of the development of T-cell repertoire among cytotoxic T-cells in the marrow chimeras [1, 2]. In this article we present data which argues that similar principles apply to T-cells which help in antibody formation, namely that MHC gene products within the thymus can influence the repertoire that emergent T helper cells possess.

In this series of studies all performed in mice $(A \times B)F_1$ stem cells were allowed to recolonise irradiated recipients of various haplotypes (A and B are used here purely as symbols for MHC disparate haplotypes). T-helper cells which developed in such chimeras were assessed for helper-function either by mixing with B-cells and antigen in an in-vitro microculture system [3, 4], or by measuring their capacity to support an in-vivo response in the chimeras to two known thymus dependent antigens GLØ[5] and (T, G)–A––L.

B. Results

I. $F_1 \rightarrow$ Parent chimeras

Chimeras of the type $(A \times B) \rightarrow F_1$ were constructed by injection of 5×10^6 anti-thy 1 and complement treated $(A \times B)F_1$ bone marrow cells into irradiated (875 r) A or B recipients [5]. These mice were primed with keyhole limpet haemocyanin (KLH) and T-helper function assessed by mixing in vitro with A or B B-cells isolated from TNP-OVA primed donors and the mixture challenged with TNP-KLH. Results for four strain combinations are given (Tables 1–3). In three combinations (Tables 1 and 2) $(A \times B)F_1$ T-helper cells which had matured in A recipients showed marked preference for cooperation with B-cells of haplotype similar to that of the recipient (i.e. A). In one combination (Table 3) cooperation was observed with B-cells derived from either parental strain although preference could be seen for B-cells of recipient haplotype.

Thus the environment in which T-cells mature to helper status dictates the MHC preferences they ultimately exhibit. The degree of MHC preference would

T-cells		Total anti TNP PFC with the following B-cells	
		C57BL/10 (H-2^b)	B10.A (H-2^a)
(B10×A/J)F_1→B10 donors 1 and 2		3,925	1,105
(B10×A/J)F_1		589	1,211
None		14	17
(B10×A/J)F_1→B10 donor	3	4,422	840
	4	194	53
	5	1,318	253
	6	1,995	253
(B10×A/J)F_1→A J donor	1	32	1,723
	2	0	1,350
	3	306	1,381
	4	498	944
(B10×A/J)F_1 pool	1	1,852	1,555
	2	1,523	1,500
None		22	14

Table 1. Restricted helper function of T cells from F_1→ parent chimeras in vitro [6] (i) T-cells were prepared from the spleens of primed and boosted (B10×A/J)F_1; (B10×A/J)F_1→ B10 and (B10×A/J)F_1→ A/J mice, and tested for helper function on B10 and B10.A B-cells. T-cells from all responding chimeric donors showed preference for cooperation with B-cells of similar haplotype to the recipients in which F_1 bone marrow cells matured. Each figure represents the total PFC (direct+indirect) from 30 microculture wells

seem to depend upon the particular strain combinations studied. Our favoured interpretation for the apparent lack of marked restriction in the data of Table 3 is that there may be one way cross reactions between the restricting loci 1-A^k and 1-A^d for helper activity to KLH. Such 'cross reactions' may be totally different when other antigens are used; but we have no information on this point.

Source of T cells	Total PFC with the following B-cells	
	CBA (H-2^k)	Balb/c (H-2^d)
Expt. 1		
(Balb/c×C3H/He)F_1→ C3H/He	2,731	86
Donor 1	2,731	86
2	2,065	108
3	2,825	142
(Balb/c×C3H/He)F_1 pool	3,181	912
None	108	83
Expt. 2		
Donor 4	1,725	0
5	3,131	89
(Balb/c×C3H/He)F_1 pool	4,184	5,751
None	110	58

Table 2. Restricted helper function from F_1→ parent chimeras in vitro [4] (ii) T-cells were prepared from primed and boosted (Balb/c×C3H/He)F_1→ C3H/He or (Balb/c×C3H/He)F_1 mice and tested for in vitro helper function on CBA and Balb/c B-cells. (Conclusions as in Table 1)

Table 3. Helper activity of T-cells of F_1→parent chimeras (iii). As in Table 2, except that (Balb/c×C3H/He)F_1→Balb/c chimeras were studied. Preference is seen for cooperation with Balb/c B-cells, although substantial helper function was detectable with CBA B cells [4]

T cells		Number	Total anti-TNP PFC with the following B-cells	
			CBA	Balb/c
Expt. 1				
None			110	58
(Balb/c×C3H/He)F_1		1×10^5	4,184	5,751
(Balb/c×C3H/He)F_1→Balb/c donor	1	1×10^5	2,882	7,526
	2	1×10^5	3,282	7,160
	3	1×10^5	2,863	8,203
	4	1×10^5	3,410	9,547
Expt. 2				
None			112	249
(Balb/c×C3H/He)F_1		1×10^5	4,609	1,991
		5×10^4	2,843	1,435
(Balb/c×C3H/He)F_1→Balb/c donor	5	1×10^5	822	2,175
		5×10^4	515	1,144
	6	1×10^5	649	1,420
		5×10^4	361	988

II. Thymus Determines the MHC Preference of T-Helper Cells

Subsequent studies [7] showed that MHC gene products within the recipient thymus were sufficient to determine the MHC preferences of T-helper cells. This was established by grafting thymus from various sources into adult thymectomised irradiated and bone marrow reconstituted ($A\times B$)F_1 recipients (Table 4).

III. The Thymus Influences MHC-Linked Ir Gene Phenotype as Measured Antibody-Formation

It is generally considered that the capacity of T-cells to help in antibody formation is in some way controlled by the MHC-linked Ir genes. Such genes have been mapped to the I-region of the mouse H-2 complex. They give the impression of being antigen-specific because strains which are nonresponders to a certain antigen are good responders to many others. It has been suggested that I-region encoded Ir genes are expressed at the level of peripheral antigen processing and presentation, and that responsiveness reflects T-cell recognition of processed antigen in the context of I-region encoded molecules on antigen presenting cells. In the following experiments we show that this recognition is also influenced by MHC gene products in the thymus.

Table 4. Thymus deterimines MHC restricted specificities of T-helper cells

Source of thymus graft		Total no. of anti-TNP PFC with the following B-cells	
		CBA(H-2^k)	Balb/c(H-2^d)
Expt. 1[a]			
C3H/He	donor 1	1,676	43
	2	1,902	0
Balb/c	donor 1	367	734
	2	996	3,836
(C3H/He × Balb/c)F_1	donor 1	1,282	1,823
	2	1,784	1,068
No T cells		83	52
Expt. 2[a]			
C3H/He	donor 3	315	0
Balb/c	3	77	1,570
No T cells		120	97
Expt. 3			
C3H/He	donor 4	2,337	205
	5	2,447	207
Balb/c	donor 4	1,121	1,943
	5	430	419
(C3H/He × Balb/c)F_1	donor 3	1,804	580
No T cells		567	30
Expt. 4			
C3H/He	donor 6	1,742	320
	7	4,346	321
Balb/c	donor 6	789	1,681
(C3H/He × Balb/c)F_1	donor 4	2,047	1,646
No T cells		324	437

[a] The efficiencies of responses of Balb/c and CBA B-cells were compared in expts. 1 and 2 by the addition of primed T cells of pools of (C3H/He × Balb/c)F_1 mice. In expt. 1 Balb/c B cells gave 2,818 PFC (in 30 wells) while CBA B cells gave 2,215 PFC. In expt. 2 BALB/c B cells gave 2,469 PFC while CBA B cells gave 1,831 PFC. (For further details see ref. [7]

IV. The Response to GLØ[5]

The response to the polymer poly-Glu^{57}-Lys^{38}-Phe^5 ($GLØ^5$) is inherited as a Mendelian dominant character [8]. It has been established that at least two complementary H-2 linked Ir genes determine GLØ responsiveness [9]. These genes have been located to the 1-A and I-E/C subregions of the mouse H-2 complex. In the chosen example the two parental strains B10 and A/J are non-responders whereas the F_1 hybrids are responders. Bone marrow cells from F_1 donors are genotypically responder. We can ask the question, whether the

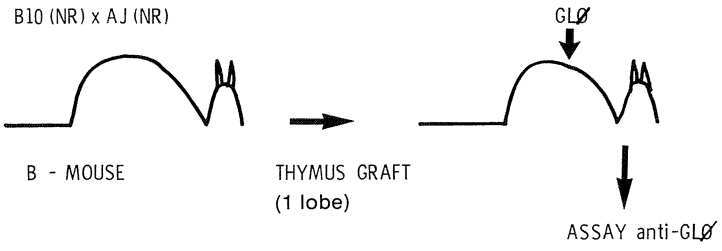


Fig. 1. Scheme for construction of thymus chimeras for examining the influence of thymus haplotype on the response of $(B10 \times A/J)F_1$ cells to GLØ

genotype of the thymus influences the capacity of F_1 T-cells to achieve responder status. To do this $(B10 \times A/J)F_1$ mice were thymectomised, lethally irradiated and reconstituted with $B10 \times A/J)F_1$ bone marrow depleted of mature T-cells using anti Thy 1 and complement. These F_1 B-mice were recipients of thymus grafts from either of the parental strain as well as from F_1 donors (Fig. 1). All thymus grafts were irradiated with 600 rads prior to grafting. Two months after grafting the animals were injected with 100 μg of in Freund's Complete Adjuvant GLØ and boosted 3 weeks later with 100 μg of GLØ in saline. Antibody levels to GLØ were measured in a radioimmunoassay. Diluted sera from individual mice were added to wells of microtitre plates which had been precoated with GLØ; and bound antibody then quantitated with ^{125}I labelled anti-mouse antibody.

It can be seen that chimeras reconstituted with B10 or A/J thymus lobes were incapable of giving an IgG response to GLØ, whereas all seven recipients of $(B10 \times A/J)F_1$ thymus responded. As the recipients of the thymuses in this experiment are the same for each group; it follows that it is the genotype of the grafted thymus which has caused the different levels in response. Further in each group the only antigen presenting cells will be F_1 cells. Consequently we can argue that the capacity of T cells to respond to GLØ is determined by MHC gene procuts within the thymus in which the T-helper cells developed. Further the gene products involved in complementation, which have already been shown to be expressed on antigen presenting cells [11], must also be expressed in the thymus.

Somewhat similar conclusions have been reached in analysis of the anti (T,G)-A-L response of thymic chimeras with the CBA (non responder) and Balb/c (responder) combinations (Munro and Waldmann, in preparation). In other words a low responder thymus confers low responsiveness of F_1 T-cells. The implications of these experiments will be discussed in more detail elsewhere.

C. Conclusions

These experiments suggest that when $(A \times B)F_1$ T-precursor cells are allowed to mature in an A thymus they come to express, to a large extent, functions associated with the MHC haplotype of the thymus donor i.e. show MHC preference (or restriction) for A B-cells and the Ir-gene phenotype of A. It is indeed compelling to think that the phenomenon simply reflects the way in which

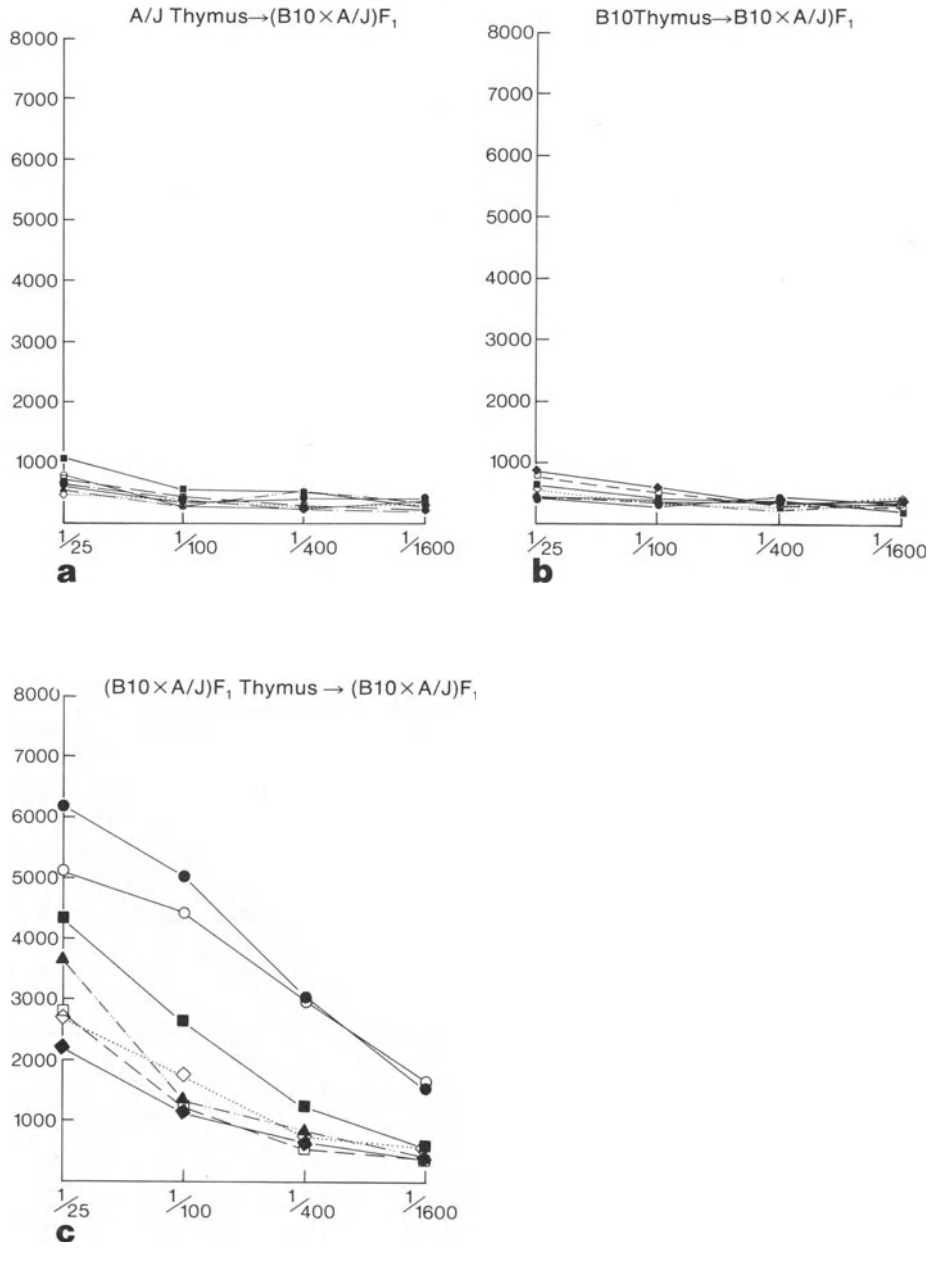


Fig. 2. Data from radioimmunoassy of sera from three groups of 7 mice which had been primed and boosted with GLØ (see text). **a** Recipients of A/J thymus, **b** recipients of B10 thymus, and **c** recipients of $(B10 \times A/J)F_1$ thymus. Abscissa = serum dilutions; Ordinate = number of counts of ^{125}I labelled antibody bound

MHC restriction is manifest. Whether or not this is true, we must conclude that the functional repertoire of T-helper cells is profoundly influenced by MHC gene products within the thymus.

We have no knowledge as yet of the selective components in the thymus that determine MHC restriction; we know that they are irradiation resistant and survive for substantial lengths of time. The thymus can be thought of as consisting of two categories of cells; immigrant cells which have migrated it into the thymus and indigenous cells which are wholly of thymic origin.

It cannot as yet be excluded that the selecting cells (selectors) are not part of the immigrant population which had seeded into the thymus. In this case in an $F_1 \rightarrow$ parent chimera the abundance of pre-existing thymic parental 'selectors' would bias the restrictions to that of the parental haplotype, whilst any new bone marrow derived F_1 immigrant selectors would only represent small proportion of those available in the thymus and their influence would be minimal.

As it stands the data would argue that bone marrow/thymus combinations for human marrow transplantation should match for HLA-D (I-equivalent) in order to develop adequate T-helper cell function. However, this assumption may be premature as a recent report has suggested that allogeneic thymuses can provide an adequate environment for developing T-cells [10]. T-cells so derived appear restricted for the MHC haplotype of the bone marrow donor and not of the thymic donor. Clearly this represents a real paradox when compared to the previous data. One possible explanation for this may be related to the origins of the selector population. In an allogeneic chimera ($A \rightarrow B$) we would expect that the indigenous thymic *(B)* selectors would generate a population of T-cells which would have no opportunity for interaction with *B* B-cells and other antigen presenting cells. Thus they would fail to be expanded in the periphery by normal environmental antigenic exposure. Any T-cells derived from intrathymic selection by immigrant *A* selectors would on the other hand expand to fill the ecological niche in the lymphoid system, and produce an effectively normal immune potential. If this were the case then our fears on mismatching for bone marrow transplantation need not be so pessimistic, at least as far as the development of immune reactivity is concerned.

Acknowledgements

We thank Heather Pope, Yvonne Pettit and Gill Kenny for their skilled assistance. This work was supported by grants from the Medical Research Council.

References

1. Zinkernagel, R. M.: Immunol. Rev. *42*, 224 (1978)
2. Boehmer, H. von, Haas, W., Jerne, N. K.: Proc. Nat. Acad. Sci. U.S.A. *75*, 2439 (1978)
3. Waldmann, H.: Immunol. Rev. *35*, 121 (1977)
4. Waldmann, H., Pope, H., Brent, L., Bighouse, K.: Nature *274*, 166 (1978)

5. Bevan, M. T.: Nature, *269*, 417 (1977)
6. Waldmann, H.: Immunol. Rev. *42*, 202 (1978)
7. Waldmann, H., Pope, H., Bettles, C., Davies, A. J. S.: Nature *277*, 137 (1979)
8. Merryman, C., Maurer, P. H., Stimpfling, J. H.: Immunogenetics *2*, 441 (1975)
9. Dorf, M. E., Stimpfling, J. H., Benacerraf, B.: J. Exp. Med. *141*, 1459 (1975)
10. Kindred, B.: Immunol. Rev. *42*, 60 (1975)
11. Schwartz, R. H., Yano, A., Stimpfling, J. H., Paul, W. E.: J. Exp. Med. *149*, 40 (1979)

Discussion

Santos: Do you have any kinetic data. You looked at 6 weeks but what about 12, 36 weeks?
Waldmann: We have taken a series of bleeds up to 3 months. The pattern has not changed in principle.
Wagner: This cross-reactivity which you find in an animal the T cells of which have been educated only in a parental type of thymus, have you already tested different haplotypes which crossreact or dont?
Waldmann: In the combination of (H-2b × H-2a) into H-2a there may to be more cross-reaction for the antigen KLH than in the reverse combination, and for CBA × Balbc into Balbc there is more cross-reaction for KLH than in the reverse combination.
Kindred: You are dealing with F-1 precursor cells which differentiate into a parental thymus. There is evidence that F1 has two different populations of helper cells. Do you think that this is a case where the F1 has essentially one population of precursors and the pattern is imposed in the thymus or is it 2 populations of precursors with selection of the preference for one depending on the type of the thymus.
Waldmann: I dont think that there are experiments which can answer the question either way. What I think is that there are two precursor types.
Slavin: Could you educate phenotypically non-responder cells to respond using a thymic graft from the responder haplotype?
Waldmann: The answer is: if the experiment is performed in the same way as the ones I have mentioned non-responder bone marrow cells which have gone through a responder thymus produce an animal which turns out to be non-responder. That might seem surprizing to you but in fact it probably is not so. The responder thymus exhibits certain MHC gene products. The non-responder cells recognize a cell. When the cells that emerge from the thymus are exposed to the antigen they would wish to interact with MHC gene products of the same hyplotype that is in the thymus, but there would be no such cells, which would bear their gene products. So the only way you can test that question, would be to transfer the cells into an F_1 which we plan to perform.

T Cell Mediated Cytotoxic Immune Reactivity of Bone Marrow Reconstituted Chimeric Mice*

H. Wagner, M. Röllinghoff, H. Rodt, and S. Thierfelder

A. Summary

The capacity of bone marrow reconstituted chimeric mice to mount sendai virus specific H-2 restricted as well as alloreactive cytotoxic T-cell responses has been investigated. Chimeras of the type $a \rightarrow (a \times b)\ F_1$ and $(a \times b)\ F_1 \rightarrow a$ were used. In addition chimeras produced with $(a \times b)\ F_1$ mice grafted under the kidney capsula with *a* type parental thymus, thymectomised, lethally irradiated and reconstituted with either *a*, $(a \times b)\ F_1$ or *b* type bone marrow cells were tested. In agreement with the data of Zinkernagel and Bevan it was noted that virus specific T cells of semi-allogeneic chimeric mice are able to recognise virus antigens in the context of the MHC of the thymus in which they have maturated. The unexpected finding was that fully allogeneic chimeras were able to mount both alloreactive CTL and H-2 restricted, virus specific CTL. The H-2 restricted CTL detected were preferentially restricted to their own MHC type. In some animals also virus specific CTL restricted to the allogeneic thymus MHC type were observed. Data are also presented to indicate that longterm cultured bone marrow cells have the potential to repopulate lethally irradiated mice.

B. Introduction

A major problem faced in allogeneic bone marrow transplantation represents the graft-versus-host (GVH) disease, a reaction known to be induced by grafted immunocompetent thymus derived (T) lymphocytes reactive to the hosts histocompatibility antigens. One of the possibilities to bypass the problem of GVH reactions is to reconstitute the recipient with hemopoetic stem cells devoid of immunocompetent T lymphocytes. Results obtained in different experimental models have indicated, that the grafted allogeneic stem cells are able to repopulate the lymphoid apparatus of the host, in which they mature into immunocompetent B and T lymphocytes specifically tolerant to the hosts histocompatibility antigens.

Recently it became clear that the gene complex coding for the major histocompatibility antigens (MHC) codes not only for the major alloantigens, but also influences T-cell responsiveness on different levels (Zinkernagel and

* This work was supported by the SFB 107 (Mainz), SFB 37 (München) and the Stiftung Volkswagenwerk

Doherty, 1977; Bevan and Fink, 1978; Gordon et al., 1977; v. Boehmer, 1977; Wagner et al., 1978). First T lymphocytes appear to recognise "foreign antigens" exclusively in the context of syngeneic MHC structures. In regard to murine cytotoxic T lymphocytes (CTL) it has been established that it is the K and the D region of the MHC which codes for the restriction specificities of CTL specific to "foreign" antigens. Second, the MHC determinants expressed on thymic epithelial cells appear to dictate the restriction specificities imposed on the maturating T cells. Third, immune response (IR) gene-controlled responsiveness of CTL appears to be linked in certain instances to MHC genes coding for the restriction elements. Fourth, if T-T cell interactions are rate limiting in the generation of antigen specific H-2 restricted CTL, MHC incompatibility between lymphocytes and the thymus may result in a loss of functional T helper cells.

Since in allogeneic chimeric mice the MHC of the lymphocytes differs from that of the hosts thymus, the question will be discussed whether T lymphocytes of successfully reconstituted chimeric mice are immunocompetent in terms of H-2 restricted CTL reactive to sendai virus antigen.

C. Material and Methods

The methodology used to produce bone marrow reconstituted chimeric mice has been described (Rodt et al., 1974). The type of chimeric mice tested includes mice of the type $a \rightarrow (a \times b)\, F_1$, $(a \times b)\, F_1 \rightarrow a$, thymectomized and lethally irradiated $(a \times b)\, F_1$ mice grafted with either *b* thymus and *b* bone marrow cells, *b* thymus and *a* bone marrow cells, $(a \times b)$ thymus and *b* bone marrow cells, or *b* bone marrow cells alone. A detailed description of these allogeneic chimeras is given by Rodt et al. (this ussue). Chimeric mice were also produced by grafting bone marrow derived stem cells cultured for 6 weeks in vitro according to the method described by Dexter et al. (1977). Spleen colony forming capacity of the cultured cells was tested by enumerating the numbers of colonies found in the spleen of lethally irradiated syngeneic recipients 9 days after transfer of a graded number of cultured stem cells. Usually 8–10 weeks after grafting the in vitro propagated lymphoid stem cells T-cell immunocompetence of the surviving animals was tested. The tissue culture system used for the induction of alloantigen specific and sendai virus specific CTL, the preparation of target cells, the ^{51}Cr cytotoxicity and the H-2 congenic and intra H-2 recombinant mice used have been described (Wagner et al., 1975; Kurrle et al., 1978).

D. Results

A prominent feature of CTL with specificity for antigens not coded for by genes of the MHC represents their dual specificity. Such CTL on the one hand are specific for foreign determinants, and on the other hand are specific for a particular self MHC structure. The phenomenon of H-2 restricted CTL has been first described by Zinkernagel and Doherty (1974). The use of intra H-2 recombinant mice soon revealed that in the CTL system the restricting elements of the self MHC structures are coded for by either the K or D region, but not by the I region of the syngeneic MHC. A typical example for the fact that in the mouse virus specific CTL can recognise virus antigens only in the context of syngeneic MHC determinants is given in Table 1. Accordingly CTL derived from sendai virus infected A. TL mice ($K^sI^dD^d$) are able to lyse sendai virus infected target cells

Table 1. H-2K or H-2D compatibility is required for cytolysis of sendai virus infected target cells

Virus specific[a] CTL	Target cells (Sendai virus)		% specific lysis[b]	H-2 compatibility
	A.TL non-	infected	2	K, I, D
	A.TL	infected	*57*	K, I, D
A.TL	C57	infected	4	none
(s kkk d)	CBA	infected	3	I
	A.SW	infected	*39*	K
	Balb/c	infected	*42*	D

[a] Splenic lymphocytes from 8 day sendai virus infected A.TL mice
[b] Ratio CTL to target cells: 20 to one; 4 hours ^{51}Cr-test; background lysis of target cells did not exceed 28%

provided the latter express either K^s or D^d histocompatibility determinants. Infected target cells expressing I^d or allogeneic histocompatibility antigens are not lysed. It should be emphasized that the phenomenon of H-2 restriction is characteristic for CTL specific to antigens not coded for by genes of the 17th chromosome; the lytic activity of alloreactive CTL is unrestricted (Billings et al., 1977; Wagner et al., 1978; Forman and Flaherty, 1978).

As soon as it was realized, that the MHC codes for the restricting elements dictating the reactivity of K or D restricted CTL, attempts were initiated to analyse whether H-2 restriction exists prior to immunisation, or whether it is imposed only as a consequence of immunisation. In regard to the problem of allogeneic bone marrow transplantation the first alternative would imply that H-2 restricted CTL to be generated are unable to see foreign antigens in the context of the hosts MHC. The basic reasoning was that if H-2 restriction is a consequence of intentional immunisation, T cells from H-2^a mice stimulated with antigen X on H-2^b cells ought to recognise antigen X only on H-2^b cells but not on H-2^a (self) cells. To bypass the problem of alloreactivity inherent to such an experimental design, this question has been studied using several models of alloreactivity. In the virus system it could be shown that T cells acutely depleted for alloreactive T cells were unable to mount virus specific CTL responses restricted to the alloantigen in question (Wagner and Röllinghoff, 1977; Bennink and Doherty, 1978). Similarly T cells from mice neonatally tolerised to a given alloantigen were able to generate virus specific CTL operating only in the context of self H-2, but not in the context of the tolerated H-2 haplotype (Zinkernagel et al., 1977). These results indicated that H-2 restriction is not a result of intentional immunisation, but is imposed prior to immunisation. Additional information was obtained using adult bone marrow chimeric mice (Pfizenmaier et al., 1976; v. Boehmer and Haas, 1976; Zinkernagel, 1976; Bevan, 1977; Zinkernagel et al., 1978a, b). For example, T cells expressing the haplotype *a* and being derived from a chimeric mouse of the type $a \rightarrow (a \times b)$ F_1 were found to lyse sendai virus infected *b* target cells after in vitro sensitisation towards sendai virus infected stimulator cells of type *b* (Table 2). The fact that normal allogeneic *b* targets were not affected by the CTL indicated that the T cells were tolerant to the alloantigen and that virus infection of the target cells was the prerequisite for cytolysis to

Table 2. Sendai virus specific lytic activity of H-2^k CTL from (CBA×C57) F_1 hybrid irradiation chimeras entirely repopulated with CBA (H-2^k) lymphocytes

Induction of effector cells			% specific lysis of ^{51}Cr-labelled targets[a]					
Responder cells	In vivo[b] treatment	In vitro stimulation	CBA (H-2^k)		C57 (H-2^b)		Balb/c (H-2^d)	
			Non infected	Infected	Non infected	Infected	Non infected	Infected
CBA	infection	CBA infected	4	*39*	5	3	2	3
CBA	infection	CBA infected	3	*47*	2	9	1	0
from CBA → (CBA×C57) F_1 chimeric mouse	infection	C57 infected	1	14	3	*36*	2	3
	infection	Balb/c non infected	–	–	–	–	*64*	–

[a] Ratio CTL to target cells 20 to one; 4 hours ^{51}Cr-test; background lysis did not exceed 31%
[b] Mice were sacrificed 10 days after in vivo infection with sendai virus

occur. The results therefore indicate that the commitment to a given restriction element is independent of the MHC genotype of the T cells; T cells from $a \rightarrow (a \times b)$ F_1 chimeric mice must have learnt during their maturation in the $(a \times b)$ F_1 environment to accept the *b* alloantigen as "self" restriction element.

It is not only that T cells can learn to accept "foreign" MHC determinants as additional self-marker, apparently the ability to accept MHC determinants as self-marker can also be lost. A typical example is illustrated in Table 3, in which data are depicted which were obtained with T cells from $(a \times b)$ $F_1 \rightarrow a$ chimeric mice. It was established that T cells from $(a \times b)$ F_1 mice contained T cells which recognize virus antigens either in the context of self antigen *a*, or in the context of self antigen *b* (Table 3). However, (CBA×C57) F_1 T cells from (CBA×C57) F_1 → C57 chimeric mice have lost their capacity to mount virus specific CTL responses in the context of CBA alloantigen (Table 3).

Table 3. Sendai virus specific lytic activity of (CBA×C57) F_1 T cells from CBA irradiation chimeras entirely repopulated with (CBA×C57) F_1 lymphocytes

Induction of effector cells			% specific lysis of ^{51}Cr-labelled targets[a]					
Responder cells	In vivo treatment	In vitro stimulation	CBA (H-2^k)		C57 (H-2^b)		Balb/c (H-2^d)	
			Non infected	Infected	Non infected	Infected	Non infected	Infected
(CBA×C57)F_1	infection	CBA – infected	2	*57*	0	14	3	1
		C57 – infected	4	19	2	*36*	0	2
(CBA×C57)F_1 from (CBA×	infection	CBA – infected	0	*31*	1	2	−3	2
C57)F_1→ CBA	infection	C57 – infected	2	1	2	3	N.D.	N.D.
Chimeric mice	infection	Balb/c – non infected	N.D.	N.D.	N.D.	N.D.	*86*	N.D.

[a] Ratio CTL to target cells: 20 to one; 4 hours ^{51}Cr-test; background lysis did not exceed 22%
Mice were sacrificed 10 days after in vivo infection with sendai virus

In an elegant analysis of the CTL responsiveness of chimeric mice grafted with thymus organs differing at the MHC with the host, Zinkernagel et al. (1978), and Bevan and Fink (1978), were able to provide strong evidence, that it is the thymus which determines the restriction specificities of H-2 restricted CTL precursors. Thus, heterozygous ($a \times b$) F_1 T cells derived from stem cells differentiating in an *a* thymus were reported to contain *a*-restricted but not *b*-restricted T cells, while homozygous *a* T cells differentiating in an ($a \times b$) F_1 thymus were found to contain both *a*- and *b*-restricted T cells. To experimentally confirm this observation, we have chosen the following test protocoll. F_1 ($a \times b$) mice were thymectomised, lethally irradiated and (1) grafted with *a*-type bone marrow cells and *a*-type neonatal thymus, or (2) grafted with ($a \times b$) F_1 type bone marrow cells and *a* type of neonatal thymus, or (3) grafted with *b*-type bone marrow cells and *a*-type neonatal thymus, or (4) grafted with *a*-type bone marrow cells alone. Two months after reconstitution the chimeric mice were sensitized with UV light inactivated sendai virus and 10 days later their T cell immunocompetence was tested in vitro. Part of the results obtained are given in Table 4. They clearly indicate that it is the H-2 genotype of the radioresistant portion of the grafted thymus, which determines the restriction specificities of the maturating T lymphocytes. Since in a given lymphoid cell population the commitment of a given restriction element seems to be independent of its own MHC genotype, it appears that it is within the thymus where the lymphoid stem cells are selected and expanded, which are capable to accept the MHC of the thymus as restriction element.

A more detailed analysis of the H-2 compatibility requirement between lymphopoetic stem cells and the thymus for generating immunocompetent CTL precursors by Zinkernagel et al. (1978a, b) and Bevan and Fink (1978) has revealed, that fully H-2 incompatible bone marrow chimeras failed to generate

Table 4. Sendai virus specific lytic activity of T cells from thymectomised (CBA × C57)F_1 irradiated chimeras grafted with irradiated neonatal thymus and entirely repopulated with either CBA or with C57 lymphocytes

Characteristics of chimera[a]				% specific lysis of target cells[b]					
Mouse	Thymus graft	Bone marrow cells	Sensitising antigen	CBA (H-2^k)		C57 (H-2^b)		Balb/c (H-2^d)	
				Non infected	Infected	Non infected	Infected	Non infected	Infected
Thymectomised (CBA × C57)F_1	–	CBA	CBA inf.	1	2	−3	0	N.D.	N.D.
			Balb/c	N.D.	N.D.	–	–	−3	1
(CBA × C57)F_1	CBA	CBA	CBA inf.	2	*27*	3	1	2	N.D.
			C57 inf.	0	1	2	−3	1	N.D.
(CBA × C57)F_1	(CBA × C57)F_1	CBA	CBA inf.	1	*19*	0	0	N.D.	N.D.
			C57 inf.	2	1	−2	*31*	N.D.	N.D.
(CBA × C57)F_1	C57	C57	C57 inf.	0	2	1	*23*	N.D.	N.D.
			CBA inf.	1	−3	4	−2	N.D.	N.D.

[a] Mice were first immunised by i.p. injection of UV-light inactivated sendai virus. After 10 days splenic lymphocytes were immunised in vitro towards the "sensitising antigen" listed

[b] Ratio CTL to target cells was 20 to one

significant levels of virus specific CTL. In fact, sharing of the I-A subregion plus either K or D was required for the induction of virus specific CTL (Zinkernagel et al., 1978). Interestingly, T cells from H-2 incompatible chimeras responded to third-party alloantigens (Dauphinee and Nordin, 1974) indicating that from a functional point of view there exists a selective block for the development of H-2 restricted T cells. Since in the studies of Zinkernagel et al. (1978) besides the K or D region also I-A region compatibility between stem cells and thymus appeared to be essential for the generation of virus specific CTL, the lack of immunocompetence in fully allogeneic chimeras has been explained with a lack of functional help.

It is with the allogeneic chimeric mice, that our results differ from those reported by Zinkernagel et al. (1978). We have tested a total of 7 fully allogeneic chimeras of the following type: (CBA×C57) F_1 mice were first grafted with a neonatal CBA thymus under the right kidney capsula, than these mice were thymectomized. 10 days later the animals were lethally irradiated (900 R) and received 20×10^6 C57BL/6 bone marrow cells treated twice with anti Thy 1.2 serum plus complement. After additional 8 weeks the reconstituted allogeneic chimeras were tested for their immune competence to mount in vitro secondary sendai virus CTL responses. The chimeric mice tested were derived from two independently produced groups. Representative results obtained are given in Table 5. Accordingly all mice tested were able to mount H-2 restricted sendai virus specific CTL in vitro. In addition upon stimulation with third-party alloantigen, alloreactive CTL could also be induced. While T cells from all allogeneic chimeras tested were able to generate alloreactive CTL, three patterns of H-2 restricted CTL responses specific for sendai virus were observed: First,

Table 5. Ability of H-2 incompatible chimeras to mount H-2 restricted sendai virus specific CTL responses

Responder T cells	In vitro restimulation	% specific lysis of targets[a]				
		CBA		C57		Balb/c
		Non infected	Infected	Non infected	Infected	Non infected
C57 ($H\text{-}2^b$) from thymectomised ($CBA \times C57)F_1$ mice Grafted with CBA thymus and C57 bone marrow	CBA infect.	3	4	2	1	−2
	C57 infect.	0	2	−1	*27*	3
	Balb/c non infect.	5	4	7	2	*43*
	CBA infect.	−1	*15*	2	6	3
	C57 infect.	3	4	2	*32*	1
	Balb/c non infect.	2	3	0	5	*51*
	CBA infect.	3	*37*	2	7	3
	C57 infect.	0	9	1	*57*	2
	Balb/c non infect.	4	6	5	9	*34*

[a] Ratio CTL to target cells 20 to one

T cells of two mice (out of seven) generated weak but significant H-2^b restricted however no H-2^k restricted CTL; second, 4 mice generated strong H-2^b restricted CTL and weak H-2^k restricted CTL; third, T cells of one allogeneic chimera generated equally efficient H-2^b and H-2^k restricted CTL. Since all mice were repopulated with H-2^b lymphocytes maturated in a H-2^k thymus, it appears that in this particular case H-2 incompatible chimeras were not immuno-incompetent, but were able to mount both alloreactive as well as H-2 restricted CTL responses.

Recently Dexter et al. (1977) have developed a system for culturing mouse bone marrow, which permits long term proliferation of stem cells able to form macroscopically visible colonies of erythroid and myeloid cells in the spleen of irradiated mice. We have investigated the potential of this culture system to support the growth of cells able to give rise to lymphocytes. The obvious rationale of our approach was, that if pluripotent stem cells grown in vitro are able to repopulate lethally irradiated mice, long term culture of bone marrow cells would be useful as a source of stem cells for transplantation when no fully matched bone marrow cells are available.

According to our experience to date, during the first week of culture of 10×10^6 bone marrow cells on a previously set bone marrow derived feeder layer the total number of non-adherent cells dropped by approximately 60–80%, after which it stabilised for 6–9 weeks at $0.3–2.5 \times 10^6$ cells per culture per week. Thus over this time period, the cultures appeared capable of continuously generating harvestable cells at the indicated rate. The non-adherent cells were Thy 1 alloantigen and surface Ig negative, and about 10–20% were peanut-agglutinin positive. The intravenous injection of graded numbers of 6 weeks-cultured cells resulted in macroscopically detectable splenic colonies almost comparable in numbers to those of normal bone marrow cells (Table 6). This finding indicated that the cultured cells retained colony forming capacity.

Table 6. Colony forming capacity of long term cultured bone marrow cells

Experimental scheme:
Bone marrow (6×10^6) → 4 weeks Tissue culture (Dexter et al.) → 9 days Irradiated host → No. of splenic colonies

Source of cells	No. of cells injected into irradiated syngeneic recipients ($\times 10^4$)	Strain of mice used[a]		
		CBA	Balb/c	C57
42 days cultured bone marrow cells	0	0, 0, 1, 0	0, 0, 0, 0	0, 0, 0, 0
	1	1, 3, 4, 12	5, 0, 3, 1	0, 2, 0, 1
	3	14, 8, 16	11, 18, 2, 4	0, 5, 2, 4
	10	15, >20, >20	9, 15, >25, >25	14, 9, 12
Normal bone marrow cells	1	6, 1, 6, 5	2, 0, 1, 4	1, 3, 2, 5
	3	10, 8, 13, 14	7, 9, 10, 7	13, 17, >25
	10	>25, >25, >25	>25, >25, 16, >25	>25, >25, >25

[a] Irradiated (850 R) mice were i.v. injected with the number of cells given. After 9 days the mice were sacrificed and the number of splenic colonies macroscopically enumerated

In a further step we used these long term cultured bone marrow cells to produce chimeric mice. So far, we have tested only a limited number of such mice. The results given in Table 7 were obtained with lethally irradiated (CBA × Balb/c) F_1 mice reconstituted with 2×10^6 non adherent cells of a 42 days old Balb/c bone marrow culture. Seven weeks after reconstitution, the mice did not have obvious signs of a GVH reaction. Their splenic lymphocytes ($55–70 \times 10^6$) expressed the H-2^d haplotype. After appropriate sensitisation, H-2^d and H-2^k restricted CTL to sendai virus infected targets could be induced. Moreover, anti H-2^b alloreactivity was found. We therefore conclude, that cells from long term bone marrow cultures appear to be suitable to repopulate lethally irradiated hosts. Further analysis of this promising approach, however, is required before the potential of its general applicability in bone marrow transplantations is warranted.

Table 7. Cytotoxic T cell immune reactivity of (CBA × Balb/c)F_1 mice fully reconstituted with Balb/c lymphocytes from in vitro long term cultured (42 days) Balb/c bone marrow cells

Responder cells	In vivo treatment	In vitro stimulation	% specific lysis of targets[a]				
			CBA		Balb/c		C57Bl/6
			Non infected	Infected	Non infected	Infected	Non infected
Balb/c lymphocytes from (CBA × Balb/c) chimeric mice fully reconstituted with Balb/c lymphoid stem cells cultured 6 weeks in vitro	Sendai virus infection	Balb/c infected	2	4	0	*34*	−2
		CBA infected	3	*27*	1	2	3
	C57	Bl/6	3	4	2	6	*63*

[a] Ratio CTL to target cells 20 to one

E. Discussion

A requirement for successful bone marrow transplantation in incompletely matched donor-recipient combinations is to avoid GVH-reactions as induced by transferred immunocompetent T cells. Recent results obtained in the mouse, however, suggest that for T cell immunocompetence to be obtained in allogeneic bone marrow transplantation, some further critical prerequisites have also to be met. According to the elegant work of Zinkernagel and associates (1977, 1978) and others (Bevan and Fink, 1978; v. Boehmer et al., 1978; Matsunaga and Simpson, 1978) certain rules appear to govern CTL responsiveness to foreign antigens. First, CTL precursors can be sensitised towards foreign antigens, such as viruses, only in the context of syngeneic MHC structures. The data available indicate that in the mouse syngeneity between CTL and targets on either the K or D region must exist for target recognition to take place. The responsiveness, however, appears to be fixed to the MHC genotype of the responder cells; it is the

MHC genotype of the thymus which imposes the restricting elements on the maturating T cells (Table 3, 4; see also Zinkernagel et al., 1978).

The unexpected finding reported here represents the observation, that allogeneic chimeras of the type $a \rightarrow b$ were capable to mount H-2 restricted CTL responses. Furthermore T cells of these mice were also able to generate in vitro alloreactive CTL. These results clearly contrast to those reported by Zinkernagel et al. (1978) and Bevan and Fink (1978), but are in accordance with those of Matzinger and Birkwood (1978). Our results raise several problems. First, Zinkernagel et al. (1978) and we ourselves (data not given) failed to generate in vivo primary H-2 restricted CTL responses in H-2 incompatible allogeneic chimeric mice. It is only in in vitro secondary responses, during which H-2 restricted CTL could be detected (Table 5). In all seven animals tested, sendai virus specific CTL restricted to the bone marrow donor H-2 type were induced. Three out of the seven animals did not generate virus specific CTL restricted to the MHC of the allogeneic thymus, three animals generated low and variable CTL activity restricted to the thymic MHC type, whereas T cells of one animal generated high and significant CTL activity restricted to the MHC of the thymus. Representative examples are given in Table 5. These results indicate that fully allogeneic chimeras contain immunocompetent CTL precursors to virus antigens. The question is, whether the immunocompetent CTL precursors are so few in numbers, that they can only be detected in in vitro secondary responses towards a ubiquitous mouse pathogen, such as sendai virus. Second, since in the allogeneic chimeras tested here H-2^b stem cells have undergone T-cell maturation in an H-2^k thymus, why did we observe primarily H-2^b restricted sendai virus specific CTL? Is it because the grafted bone marrow cells derived from H-2^b mice have undergone a prethymic differentiation step in favour of H-2^b restrictedness to be realised in an allogeneic H-2^k thymus? Alternatively, postthymic maturation in an H-2^b lymphoid cell environment could be of advantage to develop H-2^b restricted immunocompetent T cells. Third, our results contrast to the currently favoured view, that the MHC phenotype of the radioresistant portion of the thymus parallels the restriction elements accepted by the maturating T cells. Could it be, that H-2^b restricting elements crossreact so much with H-2^k MHC determinants, that the crossreactive clones within the H-2^b lymphoid cells are positively selected by the H-2^k thymic epithelium togehter with those clones capable to accept H-2^k as self restricting elements. According to this view two distinct cell populations of H-2^b phenotype may have been positively selected within the H-2^k thymus: First those which are capable to accept only H-2^k determinants as restricting elements and second a crossreactive cell population. The second cell population is thought to crossreact between H-2^k and H-2^b restricting elements. Since upon antigen exposition the first H-2^b cell population would be unable to interact with each other because of their H-2^k restrictedness, phenotypically manifested immunocompetence would be carried out primarily by the second, i.e. the crossreactive, cell population. It is only in the latter case that efficient cell interaction between virus-specific T-helper-cells and precursors of virus specific T-killer-cells would take place. It is conceivable that in secondary responses, the crossreactive CTL clones are expanded and thus are detectable. Consequently the CTL responsiveness of fully H-2 compatible chimeras is

expected to depend on the "crossreactivity" of the restriction elements of various H-2 haplotypes. This reasoning, however, does not explain, why we observed in some chimeras also H-2^k restricted CTL (Table 5).

Whatever the mechanism operating turns out to be, our results indicate that fully allogeneic chimera of the type used here are immunocompetent in respect to their capacity to mount H-2 restricted as well as alloreactive CTL responses. Obviously we now have to test, whether the particular H-2 haplotypes used here are critical for the positive results obtained. Furthermore, the importance and the influence of the secondary in vitro challenge for detecting H-2 restricted CTL has to be investigated.

A prospect of our data relevant for bone marrow transplantation is that allogeneic bone marrow transplantation may result in immunocompetence on the level of both alloreactive and H-2 restricted CTL. An other aspect of the experiments reported refers to the capacity of long term in vitro propagated bone marrow cells to confer immunocompetence to lethally irradiated mice. As evidenced in Table 6, the splenic colony forming capacity of Balb/c bone marrow cells, grown for 42 days in vitro, was almost equal to that of fresh bone marrow cells. These results hint at the possibility to use in the mouse in vitro grown lymphoid stem cells devoid of immunocompetent T cells as source of donor material in bone marrow transplantation (Table 7). On the other hand, long term in vitro grown lymphoid stem cells represent a convenient material to study in vitro the differentiation requirements of lymphoid stem cells programmed to develop into immunocompetent T lymphocytes.

Acknowledgement

The technical assistance of Ms. L. Ptschelinzew and Ms. C. Hardt is gratefully acknowledged.

References

Bennink, J. R., Doherty, P. C.: T-cell populations specifically depleted of alloreactive potential cannot be induced to lyse H-2-different virus-infected target cells. J. Exp. Med. *148*, 128–135 (1978)

Bevan, M. J.: In a radiation chimera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. Nature *269*, 417–418 (1977)

Bevan, M. J., Fink, J. P.: The influence of thymus H-2 antigens on the specificity of maturating killer and helper cells. Immunol. Rev. *42*, 3–19 (1978)

Billings, P., Burakoff, S., Martin, E. D., Benacerraf, B.: Cytotoxic T lymphocytes specific for I region determinants do not require interactions with H-2K or D gene products. J. Exp. Med. *145*, 1387–1392 (1977)

Boehmer, H. von: The cytotoxic immune response against male cells: control by two genes in the murine major histocompatibility complex. Basel: Institute of Immunology 1977

Boehmer, H. von, Haas, W.: Cytotoxic T lymphocytes recognise allogeneic tolerated TNP-conjugated cells. Nature *261*, 41–142 (1976)

Boehmer, H. von, Haas, W., Jerne, N. K.: Major histocompatibility complex-linked immune-responsiveness is acquired by lymphocytes of low responder mice differentiating in thymus of high-responder mice. Proc. Natl. Acad. Sci. USA *75*, 2439–2442 (1978)

Douphinee, M. J., Nordin, A. A.: Studies of the immunological capacity of germ-free mouse radiation chimeras. IV. Cell-mediated immunity. Cell. Immunol. *14,* 394–401 (1974)
Dexter, T. M., Allen T. D., Lajtha, L. G.: Conditions controlling the in vitro proliferation of hemopoetic stem cells in vitro. J. Cell. Physiol. *91,* 335–342 (1977)
Forman, J., Flaherty, L.: Identification of a new CML target antigen controlled by a gene associated with the Q locus. Immunogenetics, *6,* 227–23 (1978)
Gordon, R., Samelson, L., Simpson, E.: Selective response to H-Y antigen by F_1 female mice sensitized to F_1 male cells. J. Exp. Med. *146,* 606 (1977)
Kurrle, R., Röllinghoff, M., Wagner, W.: H-2 linked murine cytotoxic T-cell responses specific for sendai virus infected cells. Eur. J. Immunol. *8,* 910–812 (1978)
Matsunaga, T., Simpson, E.: H-2 complementation in anti-H-4 cytotoxic T-cell responses can occur in chimeric mice. Proc. Natl. Acad. Sci. USA *75,* 6207–6210 (1978)
Matzinger, P., Mirkwood, G.: In a fully H-2 incompatible chimera, T cells of donor origin can respond to minor histocompatibility antigens in association with either donor or host H-2 type. J. Exp. Med. *148,* 84–92 (1978)
Pfizenmaier, K., Starzinski-Powitz, A., Rodt, H., Röllinghoff, M., Wagner, H.: Virus and trinitrophenol hapten-specific T-cell mediated cytotoxicity against H-2 incompatible target cells. J. Exp. Med. *143,* 999–1004 (1976)
Rodt, H., Thierfelder, S., Eulitz, E.: Anti-lymphocytic antibodies and marrow transplantation. III. Effect of heterologous anti-brain antibodies on acute secondary disease in mice. Eur. J. Immunol. *4,* 25–29 (1974)
Wagner, H., Götze, D., Ptschelinzewe, L., Röllinghoff, M.: Induction of cytotoxic T lymphocytes against I region coded determinants: in vitro evidence for a third histocompatibility locus in the mouse. J. Exp. Med. *142,* 1477–1487 (1975)
Wagner, H., Röllinghoff, M.: T-cell mediated cytotoxicity to virus infected cells. Behring Inst. Mitt. *61,* 31–49 (1977)
Wagner, H., Starzinski-Powitz, A., Röllinghoff, M.: In: The pharmacology of immunoregulation, Werner, G. H., Flach, F. (eds), pp. 35–42. New York, London: Academic Press 1978
Wagner, H., Starzinski-Powitz, A., Röllinghoff, M., Golstein, P., Jakob, H.: T-cell-mediated cytotoxic immune responses to F9 teratocarcinoma cells: Cytolytic effector T cells lyse H-2-negative F9 cells and syngeneic spermatogonia. J. Exp. Med. *147,* 251–264 (1978)
Zinkernagel, R. M.: Virus specific T-cell mediated cytotoxicity across the H-2 barrier "virus-altered alloantigen". Nature *261,* 140–141 (1976)
Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, J., Klein, P. A., Klein, J.: On the thymus in the differentiation of "H-2 self-recognition" by T-cells: Evidence for dual recognition? J. Exp. Med. *147,* 882–896 (1978)
Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, S., Steilein, J. W., Klein, J.: The lymphorecticular system in triggering virus-plus-self-specific cytotoxic T cells: Evidence for T help. J. Exp. Med. *147,* 897–911 (1978)
Zinkernagel, R. M., Callahan, G. N., Steilein, J. W., Klein, J.: Neonatally tolerant mice fail to react against virus-infected tolerated cells. Nature *266,* 837 (1977)
Zinkernagel, R. M., Doherty, P. C.: Restriction of in vitro T-cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature *248,* 701–702 (1974)
Zinkernagel, R. M., Doherty, P. C.: The concept that surveillance of self is mediated via the same set of genes that determines recognition of allogenic cells. Cold Spring Harbor Lab. *XLI,* 505–510 (1977)

Discussion

van Bekkum: CTL is an immune response. In radiation chimeras when one finds positive responses that is a very significant result, when however you find negative responses, in my opinion this does not mean a thing because you may be dealing with subclinical graft-versus-host disease. Did you demonstrate complete chimerism?
Wagner: Chimerism was checked by complement dependent anti-H-2 sera and was 95% positive.

Waldmann: Cathy Sullivan has been looking at allogeneic chimeras with regard to possible suppression mechanisms and a preliminary data is that she could find very strong suppression mechanisms which result from apparent alloreactivity, but these had to be elicited by restimulation to become apparent.

Wagner: We have studied this question a year ago in collaboration with Dr. Rodt and Dr. Thierfelder in the F_1 chimeric situation and we looked over the time period of 2 weeks after reconstitution till 8 weeks and we were never able to find any suppressive mechanisms in these mice.

Involvement of the HLA System in the Immune Response

B. Søeberg, E. Dickmeiss, Grete S. Hansen, and A. Svejgaard

A. Introduction

Within recent years it has become clear that the major histocompatibility complex (MHC) plays a fundamental biological role in the cooperation between various cells in the immune responses. Most of this knowledge derives from studies of animals and has been summarized in recent reviews by Benacerraf and Germain (1978), Rosenthal (1978), Snell (1978), Thomas et al. (1978), and Zinkernagel (1978). It appears that MHC is involved primarily, perhaps exclusively, in thymus-dependent immunity, i.e. in cell-mediated immunity and usually in the induction of IgG antibody production. More specifically, various MHC factors play a role in the cooperation between the cell types responsible for thymus-dependent immunity: macrophages, T-helper lymphocytes, T-suppressor lymphocytes, T-effector lymphocytes, and certain B lymphocytes. A simplified scheme of these cooperations is given in Table 1 which also indicates which MHC factors seem to be involved in the various steps. In the early stages of the immunization, macrophages cooperate with T-helper lymphocytes and I region MHC determinants play a role here. These determinants are also involved in the cooperation between T-helper lymphocytes and B lymphocytes. In contrast, it is the H-2D and H-2K antigens which must be recognized on virus-infected or hapten-conjugated target cells when these are lysed by T-effector lymphocytes. Finally, the IJ antigens have been implicated in the action of suppressor cells.

For the human situation much less information is available mainly because many experiments which are easily performed in animals cannot be done in man. However, within the last two years, various observations have been reported which provide circumstantial evidence that the HLA system plays a similar role in thymus-dependent immunity in man as the H-2 and related systems do in mice and other animals. The purpose of this report is to summarize these observations

Table 1. MHC determinants involved in cellular cooperation

Cooperating cells	H-2 region determinants	HLA homologues
Macrophages/T-helper cells	I	HLA-D/DR
T-helper cells/B lymphocytes	I	?
Target cells/T-effector cells	D and K	HLA-ABC
Suppressor cells	IJ?	?

in man and to discuss some of our most recent findings in relation to in vitro studies of lymphocytes from DNCB (dinitrochlorobenzene) sensitized individuals.

B. Associations Between HLA Antigens and the Response to Immunization

The HLA system controls alloantigens, some components of the complement cascade, and most probably a variety of immune responses. There are two categories of HLA alloantigens: HLA-ABC which are present on all nucleated cells, and HLA-D/DR antigens which are mainly present on macrophages and B lymphocytes. The HLA-DR antigens are the homologues of the Ia (immune region associated) antigens in mice and are supposed to be closely related (if not identical) to the immune response (Ir) determinants. The HLA system is characterized by a pronounced linkage disequilibrium between various HLA factors, and even if we cannot as yet "type" for Ir determinants as such, there are good reasons to assume that these determinants are in linkage disequilibrium with known HLA factors. Accordingly, the existence of human Ir determinants might be reflected in associations between known HLA antigens and high and/or low responses to certain antigenic stimuli, e.g. vaccines. A number of studies aimed at detecting such relationships have been reported, and most of them have failed to show definite associations which may be due to the use of complex antigens which are not well suited to disclose *Ir* gene control. However, some of the findings are quite suggestive and interesting. For example, following vaccination an association has been reported between HLA-Cw3 and a low response to vaccinia virus in the lymphocyte transformation test (Vries et al., 1977), and Sasazuki et al. (1978) found a similar (though less significant) association between the HLA-DOH antigen and low response to tetanus toxoid in vitro after immunisation. Moreover, these authors provided evidence that the association may be due to the formation of suppressor cells (Sasazuki et al., 1978; Sasazuki, personal communication). Thomsen et al. (1975) have shown earlier that anti-adrenal auto-antibodies are significantly more frequent in HLA-Dw3 positive than in HLA-Dw3-negative patients with idiopathic Addison's disease. This observation supports the assumption that many of the HLA-D associations seen for various diseases may be due to Ir determinants. This type of research is likely to provide more positive results when HLA-D and/or DR typing become more widely used.

C. Cooperation Between Monocytes and T Lymphocytes

Independently, two groups of investigators have studied the importance of HLA antigens in the secondary immune response to foreign antigen in vitro, and they even used the same foreign antigen: PPD (=purified protein derivate=tuberculin). T lymphocytes from individuals who have been vaccinated with BCG respond by proliferation when exposed to PPD in vitro. Earlier experiments by Hansen et al. (1977) demonstrated that monocytes must be present among the lymphocytes for an optimal response to occur. Bergholtz and Thorsby (1977)

found a better cooperation between monocytes and T lymphocytes when they shared HLA-B and/or HLA-D antigens while Hansen et al. (1978) demonstrated that it is the sharing of HLA-D antigens which are of importance while the HLA-B antigens are irrelevant. This was supported by a subsequent series of experiments reported by Bergholtz and Thorsby (1978) who also studied the inhibitory action of anti-HLA-AB and anti-HLA-DR sera when these were present in the reaction mixture. They found that anti-HLA-AB sera are most active when directed against the T lymphocytes, whereas anti-HLA-DR sera are most active when directed against the HLA-D antigens shared by the monocytes and T lymphocytes. In analogy, the T-lymphocyte responses to tetanus and herpes antigen seem also to be HLA-D restricted (Hansen, unpublished).

These results confirm earlier observations in animals [6, 12] and support the assumption that HLA-D/DR antigens are important in the cooperation between macrophages and T lymphocytes and the concept that these antigens may be the Ir determinants of man.

D. Lysis of Histoincompatible, Hapten-conjugated, or Virus-infected Cells

The already classical MHC restriction phenomenon discovered by Doherty and Zinkernagel [5, 21] and by Shearer [15] was first shown to operate in man by Goulmy et al. [7]. They demonstrated that lymphocytes from a woman who was immunized against the male H-Y transplantation antigen could only lyse male target cells if these carried the HLA-A2 antigen.

Dickmeiss et al. (1977) studied the cytotoxic activity of lymphocytes from individuals sensitized to DNCB in vivo after restimulation of the lymphocytes in vitro by DNFB (dinitrofluorobenzene)-conjugated autologous lymphocytes. When subsequently tested against allogeneic DNFB-conjugated target cells, cytotoxicity was seen almost exclusively when the target cells shared HLA-A or B antigens. Again, the most effective target HLA antigen appeared to be HLA-A2, but other antigens were active as well (cf. below).

Independently of these studies, McMichael et al. (1977) provided evidence that HLA-AB antigens are also involved in the lysis of influenza virus-infected cells by lymphocytes stimulated by influenza virus-infected autologous cells in vitro. The preliminary observation that the HLA-B7 antigen seems to be superior in this process has been confirmed by McMichael (1978) in a more recent and larger series of experiments. He also provided evidence against the assumption that HLA-D antigens are involved.

Thus, the involvement of HLA-A and B antigens as part of the target for T-effector (killer) lymphocytes has been quite firmly established.

E. Recent Experiments with Lymphocytes from DNCB Sensitized Individuals

Parallel to the above summarized DNFB experiments, we have studied the proliferative response of lymphocytes from DNCB-sensitized individuals to

DNFB-conjugated autologous and allogeneic cells in vitro. Some of the results of these experiments with DNFB sensitive effector lymphocytes are summarized below. A more detailed account on the experimental protocol and the results will be published elsewhere.

Experimental Protocol

Three different protocols were followed. In all of them, peripheral blood lymphocytes from DNCB sensitized individuals were drawn at various time intervals and either used fresh or after storing in liquid nitrogen.

In the first protocol (primary cultures), lymphocytes from DNCB-sensitized individuals were cultured for various periods of time in vitro with various amounts of autologous or allogeneic DNFB-conjugated lymphocytes and the degree of lymphocyte transformation was estimated by the incorporation of isotope-labelled thymidine [17].

The second protocol (secondary cultures) consisted of first restimulating lymphocytes from DNCB-sensitized individuals with DNFB-conjugated autologous cells and then measuring the secondary (accellerated) response (transformation) to autologous and allogeneic cells some of which were coated with DNFB.

In the third protocol [4], lymphocytes from DNCB-sensitized individuals restimulated in vitro after the same principles were used as effector cells against ^{51}Cr-labelled PHA stimulated autologous and allogeneic DNFB-conjugated cells, and chromium release was used as a measure of the lytic capacity.

1. Primary Cultures

Table 2 shows the results of stimulating lymphocytes from DNCB-sensitized individuals with autologous and various allogeneic cells (unconjugated and conjugated with DNFB). It appears that autologous DNFB-conjugated lymphocytes induce a considerable degree of thymidine incorporation as compared to unconjugated autologous cells. A similar specific response is induced by DNFB-conjugated lymphocytes from two HLA identical siblings, and DNFB-conjugated cells from the father (who must share an HLA haplotype with the

Table 2. Stimulation in primary cultures of DNCB sensitive lymphocytes with autologous and allogeneic cells unconjugated and conjugated with DNFB

Relationship of stimulating cells to responder	Stimulating cells		
	Un-conjugated	DNFB-conjugated	Difference
Autologous	1,313	4,000	2,687
HLA-identical sibling: A	1,576	4,447	2,871
B	604	2,832	2,228
Father	9,309	9,698	389
Unrelated	8,204	7,302	−902

Figures are counts per minute (cpm)
The last column gives increment cpm, i.e. DNFB-conjugated minus unconjugated stimulators

responding child) also induce a slightly higher response than that seen with unconjugated cells; in this combination, unconjugated stimulator cells induce an allogeneic response due to HLA-D incompatibility. A similar allogeneic stimulation is also seen when stimulator cells not sharing HLA-D antigens with the responding cells are used. In this case, DNFB-conjugated stimulator cells induce a smaller response than unconjugated cells, and for this reason it is impossible to judge whether these cells can present DNFB to the responding cells.

However, the results obtained with HLA identical cells provide good evidence that these can present DNFB to the DNFB-sensitive lymphocytes.

2. Secondary Cultures

In preliminary experiments it became apparent that secondary cultures (i.e. using responder lymphocytes which have been primed in vitro for 10–20 days with autologous DNFB-coated cells) gave a better distinction between allogeneic DNFB-conjugated cells sharing and not sharing HLA antigens with the responding cells. Secondary cultures were used both in combinations within families and in unrelated combinations. Table 3 shows the results of one experiment in which both autologous and unrelated allogeneic cells were used as stimulators. The responses were measured after three days of the secondary culture. It appears that autologous DNFB-conjugated and allogeneic DNFB-conjugated stimulators sharing HLA-D antigens with the responder induce a rapid response. Strikingly, unconjugated allogeneic cells not sharing HLA-D antigens with the responder also induced an accellerated response which was even higher than that seen when the same cells were DNFB-conjugated. The explanation for this phenomenon is unknown. However, the accelerated response against non-conjugated cells seems to decrease after blast cell separation or prolonged pre-culture.

Nevertheless, these experiments suggest that unrelated allogeneic cells sharing HLA-D antigens with the responding lymphocytes can present DNFB for the DNCB sensitized cells. Cells sharing only HLA-A and/or B antigens with the responding cells behaved as HLA-D different cells, but here again it is difficult to rule out whether some specific DNFB presentation takes place due to the simultaneous allogeneic reaction.

Table 3. Stimulation in secondary cultures of DNCB sensitive lymphocytes with autologous and allogeneic cells unconjugated and conjugated with DNFB

Relationship of stimulating cells to responders	Stimulating cells			
	HLA-D type	Unconjugated	DNFB-conjugated	Difference
Autologous	Dw3/3	538	4,910	4,372
Unrelated	Dw3/3	1,036	7,880	6,844
Unrelated	Dw1/3	7,138	8,557	1,419
Unrelated	Dw2/2	7,638	1,842	–5,796
Unrelated	Dw4/7	8,886	678	–8,208

Figures are cpm
The last column gives increment cpm, i.e. DNFB-conjugated minus unconjugated stimulators

3. Effector Cells from DNCB Sensitized Individuals

One of the intriguing observations in our first series of experiments [4] was the apparent superiority of the HLA-A2 antigen. Accordingly, we have extended our experiments in order to substantiate the observation of preferential restriction, i.e. the phenomenon that not all of the HLA-A,B,C antigens of an individual are active in the associative recognition of DNFB-conjugated target cells. The results will be published in detail elsewhere, but Table 4 summarizes the most important observations. It appears that the HLA-A2 antigen indeed is a very effective HLA antigen in the DNFB system, but that strong lysis can also be seen in combinations involving sharing only of another HLA antigen (e.g. HLA-A1). Furthermore, in all of the individuals tested some of their HLA-A,B,C antigens did not function at all in the present system (i.e. no lysis was seen when sharing of HLA antigens between effector and target cell only involved the antigen in question). Thus the restriction phenomenon seems to be of a conservative nature.

Table 4. Lysis of DNFB-conjugated target cells

Target cells	Per cent lysis		No. of combinations
	Median	Range	
Autologous	30	25–65	10
Allogeneic			
sharing 4 Ags.	35	10–65	8
sharing 1 Ag.			
A2	45	15–65	6
A1	30	15–45	8
B5, 12 or 17	5	1–25	9
sharing O Ags.	5	1–20	18

A few combinations have been observed where weak but significant lysis of DNFB-conjugated target cells occured despite the fact that these did not share any known HLA-A,B,C antigens with the effector cells. In general, sharing of HLA-A,B,C antigens was needed for lysis of the DNFB-conjugated allogeneic cells to occur, but the exceptions nevertheless leave some doubt as to whether it is always the HLA-A,B,C antigens themselves which are involved in the target cell lysis. We have not yet investigated whether sharing of HLA-D antigens might play a role, but the many examples of strong lysis when only HLA-A antigens were shared makes it unlikely that the HLA-D antigens are necessary for the lysis itself.

F. Concluding Remarks

Although the knowledge from human experiments is much less than that from animal studies, it seems fair to state that the observations in man agree with those made in other species. In particular, the involvement of HLA-ABC antigens in the lysis by lymphocytes of histoincompatible, hapten-conjugated, and virus-infected cells seems well established, and so does the importance of HLA-D/DR

antigens for the cooperation between monocytes and T lymphocytes. However, the MHC restriction of the cooperation between T-helper lymphocytes and B lymphocytes has yet to be studied in man, and the same is true of the educational role of the thymus. Nevertheless, the recent development of plaque assays for human studies and the availability of split chimaeras after bone marrow transplantation open possibilities to study these two aspects. In general, the fact that the human species is so outbred offers special possibilities to study the MHC restriction phenomenon. For example, the preferential restriction phenomena observed for the HLA-A2 (H-Y antigen and DNCB targets) and HLA-B7 (influenza virus targets) antigens is easier to study in an outbred population. These phenomena are particularly important for our understanding of the biological function of the HLA system. With the availability of antigens such as DNFB which are less heterogeneous antigens than PPD, preferential restriction may also be observed at the level of the macrophage/T lymphocyte interaction which may offer important clues to the nature of HLA controlled immune response determinants.

Acknowledgements

The original research reported in this study was supported by grants from the Danish Medical Research Council.

References

1. Benacerraf, B., Germain, R. N.: The immune response genes of the major histocompatibility complex. Immunol. Rev. *38,* 70–119 (1978)
2. Bergholtz, B. O., Thorsby, E.: Macrophage-dependent reponse of immune human T lymphocytes to PPD in vitro. Scand. J. Immunol. *6,* 779–786 (1977)
3. Bergholtz, B. O., Thorsby, E.: HLA-D restriction of the macrophage-dependent response of immune human T lymphocytes to PPD in vitro: inhibition by anti-HLA-DR antisera. Scand. J. Immunol. *8,* 63–73 (1978)
4. Dickmeiss, E., Søeberg, B., Svejgaard, A.: Human cell-mediated cytotoxicity against modified target cells is restricted by HLA Nature *270,* 526–528 (1977)
5. Doherty, P. C., Zinkernagel, R. M.: A biological role for the major histocompatibility antigens. Lancet *1975 I,* 1406–1409
6. Erb, P., Feldmann, M.: The role of macrophages in the generation of T-helper cells. II. The genetic control of the macrophage-T-cell interaction for helper cell induction with soluble antigen. J. Exp. Med. *142,* 460–465 (1975)
7. Goulmy, E., Termijtelen, A., Bradley, B. A., Rood, J. J. van: Y-antigen killing by T cells of women is restricted by HLA. Nature *266,* 544–545 (1977)
8. Hansen, G. S., Rubin, B., Sørensen, S. F.: Human leucocyte responses in vitro. I. Transformation of purified lymphocytes with and without the addition of partially purified monocytes. Clin. Exp. Immunol. *29,* 295–303 (1977)
9. Hansen, G. S., Rubin, B., Sørensen, S. F., Svejgaard, A.: Importance of HLA-D antigens for the cooperation between human monocytes and T lymphocytes. Eur. J. Immunol. *7,* 520–525 (1978)
10. McMichael, A. J., Ting, A., Zweerink, H. J., Askonas, B. A.: HLA restriction of cell-mediated lysis of influenza virus-infected human cells. Nature *270,* 524–526 (1977)
11. McMichael, A.: HLA restriction of human cytotoxic lymphocytes specific for influenza virus. Poor recognition of virus associated with HLA-A2. J. Exp. Med. *148,* 1458–1467 (1978)

12. Rosenthal, A. S., Shevach, E. M.: The function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. J. Exp. Med. *138,* 1194 (1973)
13. Rosenthal, A. S.: Determinant selection and macrophage function in genetic control of the immune response. Immunol. Rev. *40,* 136–152 (1978)
14. Sasazuki, T., Kohno, Y., Iwamoto, I., Tanimura, M., Naito, S.: Association between an HLA haplotype and low responsiveness to tetanus toxoid in man. Nature *272,* 359–361 (1978)
15. Shearer, G. M.: Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. Eur. J. Immunol. *4,* 527 (1974)
16. Snell, G. D.: T cells, T cell recognition structures, and the major histocompatibility complex. Immunol. Rev. *38,* 3–69 (1978)
17. Søeberg, B., Andersen, V.: Hapten-specific lymphocyte transformation in humans sensitized with NDMA or DNCB. Clin. Exp. Immunol. *25,* 490–492 (1976)
18. Thomas, D. W., Clement, L., Shevach, E. M.: T lymphocyte stimulation by hapten-conjugated macrophages: a model system for the study of immunocompetent cell interactions. Immunol. Rev. *40,* 181–204 (1978)
19. Thomsen, M., Platz, P., Andersen, O. O., Christy, M., Lyngsøe, J. Nerup, J., Rasmussen, K., Ryder, L. P., Nielsen, L. S., Svejgaard, A.: MLC typing in juvenile diabetes mellitus and idiopathic Addison's disease. Transpl. Rev. *22,* 125–147 (1975)
20. Vries, R. R. P. de, Kreeftenberg, H. G., Loggen, H. G., Rood, J. J. van: In vitro immune responsiveness to vaccinia virus and HLA. N. Engl. J. Med. *297,* 692–696 (1977)
21. Zinkernagel, R. M., Doherty, P. C.: Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature *248,* 701–702 (1974)
22. Zinkernagel, R. M.: Thymus and lymphohemopoietic cells: their role in T cell maturation in selection of T cells' H-2-restriction-specificity and in H-2 linked Ir gene control. Immunol. Rev. *42,* 224–270 (1978)

Discussion

Riethmüller: With regard to the PPD system which you used there are several observations for the mouse and for man that PPD may also act as a polyclonal B cell activator.
Svejgaard: We have to be concerned with the possible polyclonal activity of PPD. It appears to go with certain batches and if you dilute your PPD to a certain extent as we did, it was a batch which did not stimulate lymphocytes from non-BCG vaccinated individuals.
Wagner: You showed preferential restriction. Does it change if you use different antigens, in other words, antigen 1 has preferential restriction to HLA-A_1 and so on.
Svejgaard: This has been shown for the H-Y and DNFB antigens. Every time lytic cells in women lyse H-Y positive cells, it is always A-2 antigen. In the DNCB system A-2 antigen was consistently better, but there were other antigens working as well.

Histocompatibility Testing for Clinical Bone Marrow Transplantation and Prospects for Identification of Donors Other than HLA Genotypically Identical Siblings

B. Dupont, R. J. O'Reilly, Marilyn S. Pollack, and R. A. Good

A. Introduction

The importance for marrow transplantation of compatibility between donor and recipient for the genetic determinants of the Major Histocompatibility Complex (MHC) has been evident since the early studies in mice (Uphoff and Law, 1958). The innoculation of immunocompetent cells into allogeneic immunoincompetent recipients leads to the development of the *graft-versus-host reaction* (GvHR). The classical GvHR model has been the injection of parental cells into F_1 hybrid mice (Simonsen, 1962; Elkins, 1971). The H-2 complex involvement in GvHR is well established.

The first indication that different components of H-2 might not be equally important in GvHR was demonstrated by Eichwald et al. 1969 and by Lengerová and Viklický, 1969. Subsequently, Démant (1970); Klein and Park (1973); Oppltová and Démant (1973); Livnat et al. (1973) and Rodey et al. (1974) demonstrated that incompatibilities at the K-end of the H-2 complex and especially I-region incompatibilities were of particular importance for GvHR. These studies supported the idea that the in vitro Mixed Lymphocyte Culture Reaction (MLR) was an in vitro model for generation of effector T lymphocytes which would parallel the in vivo GvHR.

Several of the H-2 regions are, however, capable of causing positive MLR and GvHR, and this is not limited solely to I-region differences (reviewed by J. Klein, 1975). The influence of non-H-2 differences on GvHR has been a controversial issue. Cantrell and Hildemann (1972) have, however, demonstrated that almost any minor H locus difference can induce GvHR under special conditions. The main factors involved in non-H-2 GvHR are the dose of the donor cells, preimmunization of the donor and the route of injection.

Inbred mice have provided an excellent model for the study of the fundamental factors involved in GvHR. The clinical applicability of the observations have been facilitated greatly by studies in larger, outbred animals. The first demonstration of the importance of histocompatibility testing for the MHC in bone marrow transplantation in the outbred situation was made in dogs (Epstein et al., 1968).

The present development of clinical bone marrow transplantation has clearly brought this therapy to the stage where it is the treatment of choice for an increasing number of patients with hematologic malignancies, aplastic anemia, and severe forms of primary immunodeficiency. The use of HLA genotypically identical siblings as donors of bone marrow is feasible and frequently successful. There are, however, still a large number of patients receiving this kind of

allogeneic bone marrow graft who succumb from GvHR or its associated infectious complications or who reject the marrow graft despite intensive immunosuppression prior to the transplant. The other problem of major concern to immunologists and clinicians is the fact that approximately 60–70 percent of the patients who potentially could benefit from allogeneic bone marrow transplantation do not have an HLA genotypically identical sibling as a possible donor. The present paper reviews the development of histocompatibility testing in relation to bone marrow transplantation and summarizes the few but in some instances successful attempts which have been made to extend this treatment to patients who do not have HLA identical sibling donors.

B. Genetics of HLA

I. HLA-A,B,C and D

The HLA complex is located on the short arm (p) of chromosome No. 6. It comprises approximately one thousandth of the total human genome. This chromosomal segment could contain as many as 10^5–10^6 genetic loci. Through the analysis of families with intra-HLA recombinant chromosomes, it has been shown that the HLA complex is composed of at least four major genetic loci coding for well-defined genetic determinants belonging to the same transplantation antigen system: the HLA-A,B,C and D loci (Nomenclature Commitee, 1978).

The HLA-A,B and C loci code for cell surface antigens which occur and can be serologically detected on all nucleated cells. In addition, most of these antigens are also expressed on thrombocytes. These antigens can be identified on peripheral blood lymphocytes using the complement dependent cytotoxicity test with operationally monospecific anti-HLA antibodies.

The HLA-D locus codes for genetic determinants which are responsible for stimulation in the in vitro mixed lymphocyte culture reaction. The HLA-D determinants are operationally defined using lymphocytes from HLA-D homozygous cell donors as typing reagents for these determinants. The HLA-DR determinants are serologically detected cell surface antigens that are selectively expressed on B-lymphocytes. Some of these antigens are also expressed on monocytes and on immature hemopoietic bone marrow cells. The DR antigens are analogous to the murine Ia antigens. The DR designation refers to the possibility that the DR cell surface antigens are the serological expression of the HLA-D determinant or that they code for determinants which are related to the HLA-D determinants (i.e. D related antigens).

II. The HLA-Linkage Group

Additional genes are known to be linked to HLA. The first to be identified was the gene coding for the electrophoretic polymorphism of phosphoglucomutase-3 (PGM-3). Subsequently it was found that the structural gene for factor B (Bf) of the alternative complement pathway is located close to HLA and that the genes

for complement C2 deficiency and C4 deficiency are also HLA linked. The combined data for mapping of C2 deficiency and Bf demonstrate that both genes are close to the HLA-B locus and most likely are located between HLA-B and HLA-D.

The genes coding for the two red cell antigens Chido Ch(a) and Rodgers Rg(a) are also known to be linked to HLA and these genes are also located close to HLA-B. Recent studies have shown that two HLA-B linked genes code for the polymorphic variants of C4 (the C4F and C4S loci) and that the Ch and Rg antigens are antigenic components of C4. Thus, the locus for $Ch(a)^+$ is the C4S locus and the genetic locus for $Rg(a)^+$ is the C4F locus. The gene coding for the polymorphic variants of the red cell enzyme, glyoxalase I (GLO) is located 5 Centimorgans (cm) outside the HLA-B locus (Fig. 1) (Weitkamp and Francke, 1979).

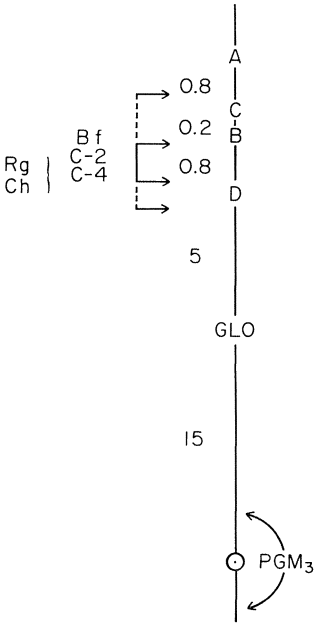


Fig. 1. The HLA linkage group

C. HLA Genotyping for Bone Marrow Transplantation

It is well recognized that the best donor choice for a potential recipient of an allogeneic bone marrow transplantation is the HLA genotypically identical sibling (Thomas et al., 1975). It is frequently not a major issue to establish HLA genotypic identity between siblings when the parents are available. There are, however, instances where the parents share one HLA haplotype or where one parent is homozygous for parts of the HLA complex. These situations sometimes require extensive studies in order to establish the correct genotypes in the family and to exclude the possibility that genetic recombinations have occurred within the HLA complex.

Other families where the parental HLA haplotypes are readily identified in the normal, healthy siblings may still pose a problem when the patients are being studied. Patients with severe combined immunodeficiency (SCID) and leukemia can sometimes be difficult to HLA type for determination of genotypic identity to one or more siblings. Extra serological reactions of patient cells may occur because of excessive numbers of Ia (DR) antigen positive B-cells, null cells or leukemic cells in the patient's circulation, which react with antibodies to DR alloantigens that co-exist in many HLA-A,B,C typing sera (Noreen et al., 1977; Pollack and Dubois, 1977). Patients with severe combined immunodeficiency are sometimes also difficult to evaluate for D locus compatibility with in vitro Mixed Lymphocyte Culture Reaction (MLR) tests, because of lack of responding or stimulating cells, while patients with acute leukemia sometimes show stimulation of HLA identical siblings in MLR presumably as a result of stimulation by "leukemia-associated" antigens (Fefer et al., 1976). Lack of in vitro lymphocyte responses or lack of MLC stimulation capacity is also seen in some leukemic patients who have received intensive chemotherapy.

The two HLA linked genetic markers: GLO and Bf provide additional tools for the identification of HLA genotypically identical siblings.

The GLO marker can be particularly useful in families where a patient and a sibling are HLA-A,C,B genotypically identical but MLR and DR typing is uninterpretable. This is demonstrated by the studies presented in Table 1. In this case, the parents were heterozygous 1–2 for the GLO marker. Since the patient and the HLA-A,C,B identical siblings were GLO homozygous (2–2) it could be concluded that no genetic recombination had occurred between the HLA-B locus and the locus for GLO. We have so far used this approach to exclude HLA recombination and indirectly to establish HLA-D identity between donors and recipients in two families where MLR studies and DR typings were uninterpretable (Pollack et al., 1979) and in a third family where clinical considerations required transplantation before MLR test results were available (Pollack et al., unpublished).

Table 1. HLA and GLO genotypes in family MR

Individual	Relationship	HLA AB Genotype		HLA DR Genotype	GLO Genotype
I.1	Father	A26,Bw49(w4) A25,B18(w6)	A/B	DRw3/DRw2	2/1
I,2	Mother	A3,B7(w6) A1,B8(w6)	C/D	DRw2/DRw3	2/1
II,1	Patient (MR)	A26,Bw49(w4) A3,B7(w6)	A/C	Uninterpretable	2/2
II,2	Sister	A25,B18(w6) A3,B7(w6)	B/C	DRw2/DRw2	1/2
II,3	Brother	A26,Bw49(w4) A3,B7(w6)	A/C	DRw3/DRw2	2/2
II,4	Brother	A26,Bw49(w4) A3,B7(w6)	A/C	DRw3/DRw2	2/2

GLO typing is also potentially useful for the evaluation of the significance of presently unknown genes between the HLA-D locus and the GLO locus. It is possible that incompatibility for other yet hypothetical MHC region genes outside HLA may play a role in failure of engraftment or the development of GvHR. The use of the GLO marker require that both parents are heterozygous and that the children are homozygous for the determinant (i.e. 50 per cent of $0.47 \times 0.47 = 0.22/2 = 11\%$) (Table 2). The percentage of informative random matings for Bf is approximately 10 per cent.

Although the most common factors for GvHR or graft rejection in allogeneic bone marrow transplantation using HLA identical sibling donors may not be HLA linked, it is very important to establish that a given transplant did involve HLA identical siblings and that not detectable genetic recombination had occurred within the HLA genetic linkage group. The GLO marker does provide a useful marker for this purpose.

Table 2. Phenotype frequencies for GLO and Bf in caucasian donors (New York)

Total	GLO Phenotypes					
	1		1–2		2	
	n	%	n	%	n	%
209	32	15	98	47	79	38

Total	Bf Phenotypes							
	S		FS		F		$S_1S;S_1F;F_1S;F_1F$	
	n	%	n	%	n	%	n	%
174	102	59	49	28	11	6	12	7

D. Use of HLA Genotypically Different Donors

I. Background

The basic concept of searching for histocompatible donor-recipient pairs who are not HLA genotypically identical siblings evolved in the early 1970s together with the recognition that separate genetic determinants were responsible for stimulation in MLR. There was no particularly promising therapeutic alternative for patients with Severe Combined Immunodeficiency Disease (SCID) who lacked an HLA identical sibling. Such patients were for example treated with histoincompatible allogeneic bone marrow transplantation following attempts to separate the bone marrow stem cells from the mature immunocompetent cells. These results were invariably unsuccessful. (Reviewed in van Bekkum, 1972 and Buckley, 1971.)

The first deliberate attempt to base the histocompatibility requirements for allogeneic bone marrow transplantation on MLR compatibility between donor

and recipient in spite of other HLA incompatibilities were made by the group in Copenhagen (Koch et al., 1973; Dupont et al., 1973). A total of nine SCID patients have now been transplanted with allogeneic bone marrow from donors, who were not HLA genotypically identical siblings (the eight cases are summarized by Dupont et al. 1979; the ninth case is briefly described in this paper).

The minimum requirement for donor-selection has in all nine cases been that the MLR between donor and recipient was compatible. Six of the patients are long-term survivors. These results are nearly as good as the average results obtained in allogeneic bone marrow transplantation in SCID using HLA identical siblings (Bortin and Rimm, 1977). In contrast to this, no patient with SCID has so far been successfully treated with bone marrow transplantation from an MLR incompatible related or unrelated donor.

The studies in SCID have provided important new information regarding the histocompatibility requirements in allogeneic bone marrow transplantation. Seven of the transplants have involved related donorrecipient pairs, and in five instances they differed for one or more HLA-A or HLA-B antigens. Four of the five patients are long-term survivors. It is evident from these studies in SCID, that HLA-D compatibility between donor and recipient is a good prediction of successful outcome of allogeneic bone marrow transplantation (Dupont et al., 1979).

II. Immunogenetic Principles for Donor Selection

Table 3 summarizes the main categories of possible bone marrow transplant donors for patients without HLA identical siblings. We have recently summarized the individually reported cases, which so far have been transplanted with bone

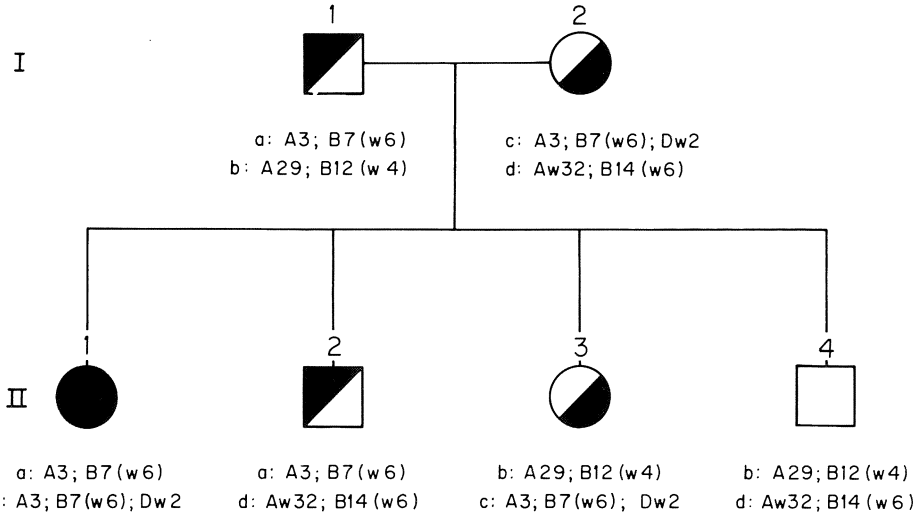


Fig. 2. Pedigree of family where the parents both have the HLA haplotype A3;B7;Dw2

Table 3. Donor selection for patients without HLA genotypically identical siblings

A. *Other relates donors*
1. HLA genotypical identity because of inbreeding
2. HLA Identity for one haplotype and:
a) Phenotypic identity for HLA-A,B,C,D on second haplotype
b) Phenotypic identity for HLA-D on second haplotype
c) Phenotypic identity for HLA-A,B,C on second haplotype
d) HLA-D homozygous recipient but HLA-D heterozygous donor
3. Other Phenotypic HLA compatible combinations
a) HLA-A,B,C,D phenotypic identity
b) HLA-A,B,C phenotypic identity
c) HLA-D phenotypic identity with varying HLA-A,B,C compatibility
B. *Unrelated donors*
1. HLA-A,B,C,D phenotypic identity
2. HLA-A,B,C phenotypic identity
3. HLA-D phenotypic identity with varying HLA-A,B,C compatibility

marrow from donors of this kind (Dupont et al., 1979). The original reports of the individual case histories are given in references Nos. 1–17 of that report.

We will not attempt to describe the individual case reports in this review but rather focus on trends which can be identified from the studies so far.

The most common bone marrow donor choice of non-HLA genotypically identical related individuals can be found in the kind of family demonstrated in Fig. 2. In this hypothetical family, the two parents share one HLA haplotype: A3;B7;Dw2. Child II,2 is HLA phenotypically identical with the mother (I,2) and Child II,3 is HLA phenotypically identical with the father (I,1). In both instances, the parents and the child are HLA genotypically identical for one HLA haplotype and are phenotypically identical for the second haplotype. As shown in Table 4, there have been seven bone marrow transplants performed with this kind of related donor. Two patients with SCID have been successfully treated (Barrett et al., 1975; Polmar and Sorensen, personal communication). In aplastic anemia, there have been three such transplants. One patient died following graft rejection (Gluckman, 1978, personal communication) and two died from GvHR (Gluckman, 1978, personal communication). Two patients with acute leukemia have been transplanted. One died from infection (Gluckman, 1978, personal communication) and one patient is a long-term survivor, but suffers from chronic GvHR (Falk et al., 1978).

Table 4 also indicates that five patients have received bone marrow transplants from HLA-D compatible donors who were incompatible for one or more HLA-A or B determinants. Three of the patients had SCID. Two of them are long-term survivors (Ramsøe et al., 1978; O'Reilly et al., 1977) and one died

Table 4. Summary of 12 bone marrow transplants performed between the first degree relatives. Rej. denotes Graft Rejection; GVH denotes Graft Vs Host Disease; Inf. denotes infection

Disease	HLA-A,B,C,D identity		HLA-D identity		HLA-A,B,C,D identity		HLA-D identity	
	$A_fB \rightarrow A_MB$ No.	Survivors	$A_fB \rightarrow A_MB$ No.	Survivors	$A_MD \rightarrow A_fD$ No.	Survivors	$A_MD \rightarrow A_fD$ No.	Survivors
SCID	2	2	2	2	0	–	1	0 GVH
Apl. anemia	1	0 Rej.	0	–	2	0 GVH	2	1 Inf. ReJ.
Acut. Leuk.	0	–	0	–	2	1 Inf. GVH	0	–
Total	3	2	2	2	4	1	3	1

from GvHR (Niethammer et al., 1976). Two patients with aplastic anemia were similarly transplanted. One of them died from infection (Opelz et al., 1978) and one survived following graft rejection but with repopulation of the marrow with autologous bone marrow cells (Territo et al., 1977).

The most recent case in this group of bone marrow transplants has been studied by our group (O'Reilly et al., 1979). A brief case report is given for this patient:

A newborn female infant was found to have an autosomal recessive Adenosine-deaminase positive form of severe combined immunodeficiency. Histocompatibility testings of the family revealed the following HLA genotypes: Patient (b/c) HLA A29,B18(w6)/A2,B8(w6); Mother (c/d): HLA A2,B8(w6)/Aw2,X; Father (a/b): HLA A1;B17(w4)/A29;B18(w6) (Table 5). In mixed lymphocyte culture, the father's lymphocytes failed to respond to those of the patient, suggesting that the mother and father (who were unrelated) shared an HLA-D specificity present on the maternal (c) and the paternal (a) haplotypes respectively (Table 6). This was also supported by analysis of primed MLC responses. Lymphocytes of the paternal grandmother (b/p) when primed against father (a/b), on restimulation, reacted as vigorously to the patient's (b/c) lymphocytes as they did to those of the father (a/b) (Table 7). At age 8 weeks, the

Table 5. HLA genotypes in SCID family JM

	HLA antigens				HLA genotypes
	–A	–C	–B	–DR	
Paternal	29	–	18(w6)	–	b
Grandmother	w31	3	40(w6)	w4	p
Father	1	–	17(w4)	w7	a
	29	–	18(w6)	–	b
Mother	2	–	8(w6)	w7	c
	w23	–	–	w3	d
Child	29	–	18(w6)	–	b
(SCID)	2	–	8(w6)	w7	c

Table 6. Mixed lymphocyte culture reactions in vitro in SCID family JM. The numbers in parentheses are the cpm obtained in autologous cell cultures. The reference value is the mean-cpm obtained when 2 single donors and 2 pools of 4 donors are used as stimulating cells. The percent values are the relative responses (RR)≤9% denotes MLR compatibility

Responder	Stimulator				cpm reference value 100%
	b/c_X	a/b_X	c/d_X	b/p_X	
Patient (b/c)	(121)	–	–	–	961
Father (a/b)	1%	(125)	60%	50%	8,916
Mother (c/d)	30%	45%	(350)	83%	11,663
Paternal GM (b/p)	26%	50%	95%	(100)	8,132

patient received a transplant of marrow from the father at a dose of 1.9×10^9 nucleated marrow cells/kg. Moderately severe graft vs. host disease involving skin, liver and intestinal tract was reversed with prednisone. The patient was discharged 8 weeks post transplant, with full immunologic function and stable mixed chimerism of lymphoid and hematopoietic elements.

1. HLA-D Homozygous Recipients

Three HLA-D homozygous patients have been transplanted with bone marrow from an HLA-D heterozygous related donor. This kind of transplant should simulate the experimental model of F_1 hybrid grafting into a parental strain. In Fig. 2, this would correspond to a transplant from one of the parents into the HLA homozygous child II,1.

The results obtained in these three transplants are summarized in Table 8. The patient with aplastic anemia reported by O'Reilly et al. (1978) was transplanted

Table 7. Secondary in vitro mixed lymphocyte culture reactions in SCID family JM. The priming cell culture is given at the top of the table where the lymphocytes from the paternal gradmother are primed against the father's cells. The priming haplotype is a: A1;B8(w6);DRw7. The maternal cells (c/d) also give strong restimulation. The results are given as percent restimulation compared with the net-cpm obtained with the specific restimulating cells $(a/b)_X$

Cell donors	Priming culture	Priming haplotype
Paternal grandmother × father	$b/p \leftarrow (a/b)_X$	a:A1;B17(w4);DRw7

	restimulation		
Secondary culture dilution Responder: stimulator	Patient (b/c) c: A2;B8(w6);DRw7	Mother (c/d) c: A2;B8(w6);DRw7 d: Aw23;–;DRw3	Unrelated
1:1	503	110	0
1:2	242	113	44
1:4	78	64	24
1:8	44	53	10

Table 8. Summary of three bone marrow transplants where the recipients were HLA-D homozygous

Disease	$A_FB \rightarrow$ No.	A_FA_M Survivors	$A_MB \rightarrow$ No.	A_FA_M Survivors
Apl. anemia	0	–	2	1
Acut. leuk.	1	0	0	–
Total	1	0	2	1

with bone marrow from the HLA-D heterozygous sister. The patient is now fully hematologically reconstituted and is chimeric more than 18 months post transplantation. The second patient with aplastic anemia, who was transplanted following the same principle died from infection on day 20 post transplantation (Opelz et al., 1978). The patient with acute leukemia studied by O'Reilly (unpublished) died from intracranial hemorrhage following the second transplant. The patient who was chimeric at that time and was not suffering from GvHR was in the early stage of reconstitution.

2. HLA Recombinant Donor-Recipient Pairs

Table 9 summarizes the 5 patients who have been transplanted with bone marrow from siblings where the donor and recipient differed for one or more HLA determinants because of intra-HLA recombination in the family. One patient with SCID is a long-term survivor following transplantation in spite of one HLA-A locus incompatibility (Gatti et al., 1968). Three patients with acute leukemia were transplanted with bone marrow from HLA-D incompatible donors, where the incompatibility was caused by an HLA-B:D recombination. One patient died on day 41 from recurrence of leukemia (Gale et al., 1975); one patient died on day 278 from GvHR (Opelz et al., 1978) and one died on day 34 from infection and GvHR (Blume et al., 1978).

Table 9. Summary of five bone marrow transplants where the HLA incompatibilities were caused by intra-HLA recombinations

Disease	HLA		Difference			
	No.	–A survivors	No.	–B survivors	No.	–D survivors
SCID	1	1	0	–	0	–
Apl. anemia	1	1	0	–	0	–
Acute leuk.	0	–	0	–	3[a]	0
Total	2	2	0	–	3	0

[a] 1 GvH died day 278 post-transplantation; 1 relapse died day 41 post-transplantation; 1 infection died day 34 post-transplantation

3. Unrelated Bone Marrow Donors

Five reported cases have received bone marrow transplants from unrelated donors. In all cases the donor has been selected primarily because of HLA-D compatibility between donor and recipient. The case reported by O'Reilly et al.

1977 with SCID is the only long-term survivor. This patient suffers from chronic GvHR primarily involving the skin. No other unrelated bone marrow transplantation has resulted in long-term survivors.

E. Summary

Clinical histocompatibility testing has now developed to a stage where it is possible to select related bone marrow donors for some patients, when HLA genotypically identical siblings are not available. The most common type of such donors are the HLA phenotypically or HLA-D phenotypically identical related donors. The HLA-D homozygous recipient offers special options, since these patients can potentially receive bone marrow transplants from any of the parents or from HLA-haploidentical siblings. The studies in SCID have demonstrated that HLA-D compatibility in spite of HLA-A or B incompatibilities can be tolerated and there is now accumulating evidence that even patients with aplastic anemia or acute leukemia can be successfully treated with such bone marrow grafts.

Acknowledgements

Original work quoted in this paper was in part supported by grants from U.S. Public Health Service CA 19267, CA 08748, CA 17404, CA 22507, CA 23766 and by the Judith Harris Selig Memorial Fund, the Arthur Vining Davis Foundations, the Charles E. Merrill Trust, the Zelda R. Weintraub Cancer Fund and by the Charles A. Dana Foundation.

References

Barrett, A. J., Humble, J. G., Hobbs, J. R.: Bone marrow suppression by antilymphocytic globulin. Br. Med. J. *2*, 541 (1975)

Bekkum, D. W., van: Use and abuse of hemopoietic cell grants in immune deficiency diseases. Transplant. Rev. *9*, 3–53 (1972)

Blume, K. G., Bross, K. J., Chillar, R. K., Findley, D. O., Opelz, G., Paladugu, R. R., Sharkoff, D., Warner, J. A.: Bone marrow transplantation between mixed leukocyte culture reactive siblings. Acta Haematol. (Basel) *59*, 257–261 (1978)

Bortin, M. M., Rimm, A. A.: Severe combined immunodeficiency disease. Characterization of the disease and results of transplantation. JAMA *238*, 591–600 (1977)

Buckley, R. H.: Reconstitution: Grafting of bone marrow and thymus. In: Progress in immunology. Amos, D. B. (ed.) pp. 1061–1080. New York, London: Academic Press 1971.

Cantrell, J. L., Hildemann, W. H.: Characteristics of disparate histocompatibility barriers in congenic strains of mice. I. Graft-versus-host reactions. Transplantation *14*, 761–770 (1972)

Démant, P.: Genetic requirements for graft-versus-host reaction in the mouse. Different efficacy of incompatibility at D- and K-ends of the H-2 locus. Folia Biol. (Praha) *16*, 273–275 (1970)

Dupont, B., Andersen, V., Ernst, P., Faber, V., Good, R. A., Hansen, G. S., Henriksen, K., Jensen, K., Juhl, F., Killmann, S. Aa., Koch, C., Muller-Bérat, N., Park, B. H., Svejgaard, A., Thomsen, M., Wiik, A.: Immunologic reconstitution in severe combined immunodeficiency with HL-A incompatible bone-marrow graft: Donor selection by mixed lymphocyte culture. Transplant. Proc. *5*, 905–908 (1973)

Dupont, B., O'Reilly, R. J., Pollack, M. S., Good, R. A.: Use of HLA genotypically different donors in bone marrow transplantation. Transplant. Proc. *11,* 219–224 (1979)

Eichwald, E. J., Hart, E. A., Eichwald, B.: Genetic aspects of the graft-versus-host reaction of mice. Folia Biol. (Praha) *15,* 254–258 (1969)

Elkins, W. L.: Cellular immunology and the pathogenesis of graft-versus-host reactions. Prog. Allergy *15,* 78–187 (1971)

Epstein, R. B., Storb, R., Ragde, H., Thomas, E. D.: Cytotoxic typing antisera for marrow grafting in littermate dogs. Transplantation *6,* 45–58 (1968)

Falk, P. M., Herzog, P., Lubens, R., Wimmer, R. S., Sparkes, R., Naiman, J. L., Gale, R. P., Koch, P., August, C., Feig, S. A., UCLA Bone Marrow Transplant Group: Bone marrow transplantation between a histocompatible parent and child for acute leukemia. Transplantation *25,* 88–90 (1978)

Fefer, A., Mickelson, E., Thomas, E. D.: Leukemia antigens. Stimulation of lymphocytes in mixed culture by cells from HLA identical siblings. Clin. Exp. Immunol. *23,* 214–218 (1976)

Feig, S. A., Opelz, G., Winter, H. S., Falk, P. M., Neerhaut, R. C., Sparkes, R., Gale, R. P., UCLA Bone Marrow Transplant Group: Successful bone marrow transplantation against mixed lymphocyte culture barrier. Blood *48,* 385–391 (1976)

Gale, R. P., Opelz, G., Sparkes, R., UCLA Bone Marrow Transplant Group: Bone marrow transplantation between mixed lymphocyte culture-reactive individuals. Transplantation *20,* 194–197 (1975)

Gatti, R. A., Meuwissen, H. J., Allen, H. D., Hong, R., Good, R. A.: Immunological reconstitution of sex-linked lymphopenic immunological deficiency. Lancet *1968 II,* 1366–1369

Horowitz, S. D., Groshong, T., Bach, F. H., Hong, R., Yunis, E. J.: Treatment of severe combined immunodeficiency with bone-marrow from an unrelated mixed-leukocyte-culture-non-reactive donor. Lancet *1975 II,* 431–433

Jeannet, M., Speck, B., Sartorius, J.: Donor selection for bone marrow transplantation. Predictive value of DR typing for mixed lymphocyte culture compatibility between unrelated individuals. Transplantation *26,* 448–449 (1978)

Klein, J., Park, J. M.: Graft-versus host reaction across different regions of the H-2 complex of the mouse. J. Exp. Med. *137,* 1213–1255 (1973)

Klein, J.: Biology of the mouse histocompatibility-2 complex. New York, Heidelberg, Berlin: Springer 1975

Koch, C., Henriksen, K., Juhl, F., Wiik, A., Faber, V., Andersen, V., Dupont, B., Hansen, G. S., Svejgaard, A., Thomsen, M., Ernst, P., Killmann, S. Aa., Good, R. A., Jensen, K., Muller-Bérat, N.: Bone marrow transplantation from an HL-A non-identical but MLC identical donor. Lancet *1973 I,* 1146–1150

Lengerová, A., Viklický, V.: Relative 'strength' of histocompatibility barriers as compared on the basis of different criteria. Folia Biol. (Praha) *15,* 333–339 (1969)

Livnat, S., Klein, J., Bach, F. H.: Graft versus host reaction in strains of mice identical for H-2K and H-2D antigens. Nature New Biol. *243,* 42–44 (1973)

Lohrmann, H.-P., Dietrich, M., Goldmann, S. F., Kristensen, T., Fliedner, T. M., Abt, C., Pflieger, H., Flad, H.-D., Kubanek, B., Heimpel, H.: Bone marrow transplantation for aplastic anaemia from a HL-A and MLC-identical unrelated donor. Blut *31,* 347–354 (1975)

Niethammer, D., Goldmann, S. F., Haas, R. J., Dietrich, M., Flad, H.-D., Fliedner, T. M., Kleihauer, E.: Bone marrow transplantation for severe combined immunodeficiency with the HL-A-A-incompatible but MLC-identical mother as donor. Transplant. Proc. *8,* 623–628 (1976)

Nomenclature Commitee: Nomenclature for factors of the HLA system 1977. In: Histocompatibility testing 1977. Bodmer, W. F., Batchelor, J. R., Bodmer, J. G., Festenstein, H., Morris, P. J. (eds.) pp. 14–20. Copenhagen: Munksgaard 1978

Noreen, H. J., Hagen, B. A. van der, Greenberg, L. J., Kersey, J., Emme, L., Yunis, E. J.: Previously unexplained HLA antigens of combined immunodeficiency disease due to Ia alloantigens. Transplant. Proc. *9,* 1717–1719 (1977)

Opelz, G., Gale, R. P., Feig, S. A., Walker, J., Terasaki, P. I., Saxon, A., UCLA Bone Marrow Transplant Team: Significance of HLA and non-HLA antigens in bone marrow transplantation. Transplant. Proc. *10,* 43–46 (1978)

Oppltová, L, Démant, P.: Genetic determinants for the graft-vs.-host reaction in the H-2 complex. Transplant. Proc. *5,* 1367 (1973)

O'Reilly, R. J., Dupont, B., Pahwa, S., Grimes, E., Smithwick, E. M., Pahwa, R., Schwartz, S., Hansen, J. A., Siegal, F. P., Sorell, M., Svejgaard, A., Jersild, C., Thomsen, M., Platz, P., L'Esperance, P., Good, R. A.: Reconstitution in severe combined immunodeficiency by transplantation of marrow from an unrelated donor. N. Engl. J. Med. *297,* 1311–1318 (1977)

O'Reilly, R. J., Pahwa, R., Kirkpatrick, D., Sorell, M., Kapadia, A., Kapoor, N., Hansen, J. A., Pollack, M., Schutzer, S. E., Good, R. A., Dupont, B.: Successful transplantation of marrow from an HLA: A,B,D, mismatched heterozygous sibling donor into an HLA-D homozygous patient with aplastic anemia. Transplant. Proc. *10,* 957–962 (1978)

O'Reilly, R. J., Sorell, M., Pollack, M., Kapoor, N., Chaganti, R. S. K., Good, R. A., Dupont, B.: Reconstitution of immunologic function in a patient with severe combined immunodeficiency following transplantation of marrow form an HLA-A,B,C non-identical but MLC compatible paternal donor. Transplant. Proc. (in press)

Pollack, M. S., Dubois, D.: Possible effects of non-HLA antibodies in common typing sera on HLA antigen frequency data in leukemia. Cancer *39,* 2348–2354 (1977)

Pollack, M. S., Yang, S. Y., O'Neill, G. J., O'Reilly, R. J., Grossbard, E., Kapoor, N., Good, R. A., Dupont, B.: Bone marrow transplantation using typing for glyoxalase-I as a tool in histocompatibility testing. Transplantation *28,* 156–158 (1979)

Ramsøe, K., Skinhøj, P., Andersen, V., Ernst, P., Faber, V., Platz, P., Thomsen, M., Svejgaard, A., Eriksen, K. R., Plesner, T., Morling, N., Philip, J., Killmann, S. Aa., Koch, C., Muller-Bérat, N., Henningsen, K., Axelsen, N. H.: Successful nonsibling bone marrow transplantation in severe combined immunodeficiency. Transplantation *26,* 369–372 (1978)

Rodey, G. E., Bortin, M. M., Bach, F. H., Rimm, A. A.: Mixed leukocyte culture reactivity and chronic graft versus host reactions (secondary disease) between allogeneic H-2^k mouse strains. Transplantation *17,* 84–88 (1974)

Simonsen, M.: Graft versus host reactions. Their natural history, and applicability as tools of research. Prog. Allergy *6,* 349–467 (1962)

Speck, B., Zwaan, F. E., Rood, J. J. van, Eernisse, J. G.: Allogeneic bone marrow transplantation in a patient with aplastic anemia using a phenotypically HLA-identical unrelated donor. Transplantation *16,* 24–31 (1973)

Territo, M. C., Gale, R. P., Feig, S., Opelz, G., Young, L., Fahey, J. L., Cline, M., Langdon, E., Juillard, G., Naeim, S., Sparkes, R., Golde, D., Haskell, C., Smith, G., Fawzi, F., Sarna, G.: Autologous bone marrow repopulation following high dose cyclophosphamide and allogeneic marrow transplantation in aplastic anaemia. Brit. J. Haematol. *36,* 305–312 (1977)

Thomas, E. D., Storb, R., Clift, R. A., Fefer, A., Johnson, F. L., Neiman, P. E., Lerner, K. G., Glucksberg, H., Buckner, C. D.: Bone-marrow transplantation. N. Engl. J. Med. *292,* 832–843 (part 1); 895–902 (part 2) (1975)

Uphoff, D. E., Law, L. W.: Genetic factors influencing irradiation protection by bone marrow. II. The histocompatibility-2 (H-2) locus. J. Natl. Cancer Inst. *20,* 617–624 (1958)

Weitkamp, L., Francke, U.: Report of the commitee on the genetic constitution of chromosome 6. In: Human gene mapping 4. Winnipeg Conference 1977 pp. 92–105. Basel: Karger 1978; Cytogenetics and Cell Genetics *22,* 1–6 (1978); Birth Defects: Original Article Series *14,* 4 (1978)

Discussion

Niethammer: I will report tomorrow, that we have indeed grafted a patient with congenital graft-versus-host disease with marrow from her HLA-A,B and D-mismatched mother. The marrow was engrafted and the patient was immunologically restored.

Dupont: I have not said that there are no transplants between completely mismatched relatives. I said that there are no long term survivors because of GVHD.

Vriesendorp: How did you define HLA-D phenotype identity?

Dupont: Less than 9 per cent relative response in mutual in vitro MLC test.

Storb: I would like to commend that we have grafted a number of patients from non-HLA-identical family members. Three of those were the result of recombinations of HLA-D with very rigorous mutual stimulation of which two patients with leukemia grafted in remission are alive at one and a half and one year post transplantation. Both did not have the slightest trace of GVHD which supports the view that HLA-D may not be directly involved in determining GVHD.

Dupont: Theoretically, there is possibility for HLA-D incompatibility without GVHD. There have been reports on combined CML and MLC studies in HLA-B-D recombinants in which the CML-locus segregated either with the HLA-D or the HLA-B complex. It is conceivable that the CML locus is more important. What I said was that there is no success reported of bone marrow transplantation across the mismatch of a whole haplotype including incompatibility for HLA-D. At least one of the cases which you refer to involved HLA-A,B,C genotypically identical siblings, where the donor and recipient differed for one HLA-D determinant because of an HLA-B:D recombinant haplotype (Family UPN 762 in R.A. Clift et al. Transplantation *28*, 235–242 [1979]).

Note added in proof: The paper by Clift et al. referred to above has occurred subsequent to this symposium. The paper describes bone marrow transplants in 12 patients with acute leukemia and nine patients with aplastic anemia. All were transplanted with donors other than HLA identical siblings. Five of the patients are long-term survivors.

T Cell Subpopulations After Allogeneic Bone Marrow Transplantation

A. Bacigalupo, M. C. Mingari, L. Moretta, M. T. van Lint, G. Piaggio, M. R. Raffo, M. Podesta and A. M. Marmont

A. Abstract

T-cell subpopulations were studied in six patiens after allogeneic bone marrow transplantation for aplastic anemia and acute leukaemia, by means of receptors for IgG (T_G-cells) and IgM (T_M-cells). Significant modifications of T_M-T_G levels were found: a 2 to 10 fold increase of $T_G$cells and a 2 to 10 fold decrease of $T_M$cells from pre-transplant levels could be observed. The T_M-T_G imbalance appeared together with the clinical signs of GVHD: the level of T_M-depression (but not the level of T_G increase) correlated with the severity of GVHD. The T_M-T_G modifications were observed as long as 3 years after transplantation, also in the absence of signs of GVHD. In addition, all six patiens were found to have severely impaired pokeweed-induced B-cell differentiation. The clinical implications and the possible lack of T-helper activity are discussed.

B. Introduction

Bone marrow transplant (T_x) recipients suffer from a prolonged period of immunodeficiency [1, 2, 8]: several in vitro and in vivo tests, comprising mitogen responsiveness, skin tests and in vitro immunoglobulin synthesis [2, 8] are abnormal for 3 months to 2 years, in spite of normal numbers of circulating T-cells.

In the attempt of elucidating the pathogenesis of the clinical immunodeficiency in transplanted patients, T-cell subpopulations were analyzed in six chimeras after purification of T cells by means of sheep red blood cell-rosettes [4] and separation of lymphocytes according to their receptors for IgG ($T_G$cells) or IgM ($T_M$cells) [5].

C. Materials and Methods

Patients were transplanted in Genova for aplastic anemia (3 patients) or acute leukemia (3 patients). Conditioning regimens consisted of cyclophosphamide 200 mg/kg for patients with severe aplastic anemia (SAA) and cyclophosphamide 120 mg/kg followed by 1000 rads total body irradiation (TBI) and bone marrow for the acute leukemias. Post-transplant immunosuppression was given according to the Seattle protocol with methotrexate (MTX). Bone marrow was always obtained from HLA A,B,C identical siblings negative in one-way mixed lymphocyte cultures (MLC). The donor origin of the circulating lymphocytes was

assessed in 5 out of 6 cases my means of cytogenetics (in opposite sex pairs). Patients experiencing clinical GVHD received additional immunosuppression consisting of high dose 6-methylprednisolone (6-MPr) or antilymphocytic globulin (ALG). Follow up periods varied from 100 to 1000 days.

Surface markers of circulating lymphocytes were studied after separation on a density gradient consisting of Ficoll-Hypaque [4]: E-rosettes, surface immunoglobulins were performed as reported elsewhere [4]. Rosettes of T cells with IgG or IgM coated ox-erythrocytes (EAIgG, EAIgM rosettes) were performed as described elsewhere [4, 7] on freshly drawn (EAIgG) or cultured (overnight) (EAIgM) purified T-lymphocytes.

Serum immunoglobulins were measured with routine immunodiffusion.

Pokeweed induced B-cell differentiation was evaluated after 7 day-cultures of 1×10^5 B cells incubated with 5×10^4 T cells [6]: the number of cells with detectable cytoplasmic Ig (cIg+cells) were scored after labelling with total anti-immunoglobulin, FITC-conjugated, serum [6].

D. Results

Table 1 summarizes the levels of sheep red blood cells-rosettes (E-rosettes), surface Ig (SIg) and serum Ig before, within 100 days and after 100 days from transplantation: no significant modifications were observed.

	Pre-Tx	Post-Tx <100 d.	Post-Tx >100 d.
SIg	8.6±6.2%	10.1±6.3%	11.2±3.3%
E-ros	68.5±4.8%	69.6±8.7%	70.1±3.1%
Serum IgG	910±413	720±514	517±42
IgA	149± 85	91± 55	54±36
mg% IgM	105± 61	102± 62	53±36

Table 1. Surface immunoglobulins, E-rosetting cells and serum immunoglobulins before, within 100 days and after 100 days from bone marrow transplantation

Table 2 represents our data on T_M and T_G levels before and after transplantation: an important decrease of T_M-cells together with an increase of T_G-cells is evident. These modifications correlated with the appearence of clinical GVHD (Fig. 1) and the level of T_M depression (unlike the level of T_G-increase) paralleled the severity of GVHD (Table 3).

T-cells	Normal %	Pre-Tx %	Post-Tx <100 d. %	Post-Tx >100 d. %
EA-IgG (T_G)	11±1	13±5	33± 9	38±10
EA-IgM (T_M)	56±5	49±5	11±12	22± 9

Table 2. Rosettes with IgG and IgM coated ox-erythrocytes before and after bone marrow transplantation: T_G increase and T_M depression in six chimeras

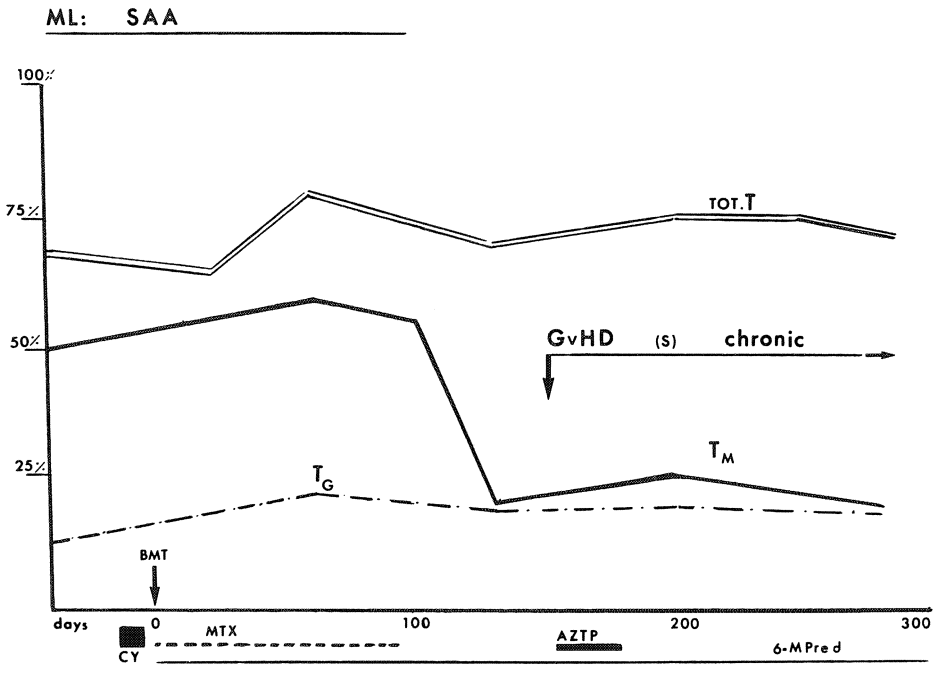


Fig. 1. T_M, T_G and total T cells before and after allogeneic bone marrow transplantation in a case of severe aplastic anemia: T_M and T_G levels remain within the mormal range untill day 120 when chronic skin GVHD appears

In Table 4 the results of PWM-induced B-cell differentiation are outlined:
a) lymphocytes from chimeras did not show differentiation to PWM as demonstrated by the lack of cells with cytoplasmic immunoglobulins (cIg+cells) after 7 days.
b) chimera's T lymphocytes did not provide adequate "help" for control B lymphocytes and again an extremely low number of cIg+cells were recovered after 7 days.
c) the depletion of chimera's T_G-cells (T_G-cells = T_M and T_{null}cells) did not release T cells from possible suppression, and cIg+cell recovery at 7 days was still very low.
d) impaired PWM-induced B cell differentiation was observed in one case at 3 years after transplantation after resolution of akute GvHD.

Table 3. EAIgG and EAIgM rosettes and GVHD: levels of $T_M$depression correlate with severity of GVHD

T cells	Normal %	GVHD 0–I %	GVHD II–III %
EA-IgG (T_G)	11±1	29±6	39±12
EA-IgM (T_M)	56±5	23±3	5± 4

T cells (5×10^4)	+B cells+PWM= (1×10^5)	cIg+cells (No./1.5×10^5 cells)
Total-T contr.	+B contr. +PWM=	$100\times10^3\pm5$
Total-T chim.	+B chim.+PWM=	$5\times10^3\pm3$
Total-T chim.	+B contr. +PWM=	$18\times10^3\pm9$
T_G-depleted chim.	+B contr. +PWM=	$12\times10^3\pm2$

Table 4. Pokeweed induced-B-cell differentiation after bone marrow transplantation: severe impairment of differentiation in chimeras, lack of "help" of chimera T cells for control B lymphocytes and persistent "lack of help" also after depletion of T_G cells

E. Discussion

Combined immunodeficiency has been well documented in patients after allogeneic bone marrow transplantation [1, 2, 8]: in spite of normal numbers of circulating T-lymphocytes several in vivo and in vitro tests demonstrate a depressed T-cell function for periods ranging from 2 months to 2 years after Tx [1, 2, 8]. Thus T cells appear to be, at least partially, inactive, though the pathogenesis for such a disfunction is still unclear: the existence of suppressor T cells and/or the lack of helper T cells may be relevant in this regard.

A method for separating helper and suppressor cells has recently been described [6]: it involves rosette formation with ox erythrocytes coated with rabbit IgG or IgM (EAIgG and EAIgM rosettes). Lymphocytes that form rosettes with IgG-coated erythrocytes are named $T_G$cells, whereas cells forming rosettes (after an overnight incubation at 37° C) with IgM-coated erythrocytes are referred to as T_M [6]. The identification of $T_M$lymphocytes as helper and of T_G cells as suppressor lymphocytes has been well documented [6].

Six chimeras were thus studied with special regard to their T cell subpopulations and important modifications of the T_M-$T_G$cells were found: T_M(helper) cells were depressed 2 to 10 fold from pre-Tx (or normal) levels; T_G(suppressor) cells were increased 2 to 10 fold from pre-Tx (or normal) levels. This finding suggested three questions: is the T_M-T_G imbalance somehow related to GVHD?, does it account for the clinical immunodeficiency? and thirdly how long does it last in the chimera?. As to the first question T_M-T_G modifications appeared together with the clinical signs of GVHD and the levels of T_M depression correlated with the severity of GVHD. As to the duration, this imbalance could be detected as long as 3 years after Tx in a patient after resolution of all clinical and biochemical signs of GVHD. Finally to answer the question of the importance of T_M depression and T_G-increase in the immunodeficiency of transplanted patients, experiments of pokeweed (PWM)-induced B-cell differentiation were set up with and without chimera T-lymphocytes: the conclusions were that

a) PWM-induced B-cell differentiation is impaired in chimeras,

b) co-culture of control B lymphocytes with chimera T-cells did not result in adequate differentiation into cIg+cells,

c) chimera T cells, depleted of T_G(suppressor)cells, still could not provide help for control B lymphocytes in response to PWM.

In conclusion we have shown that important modifications of T-cell subpopulations occur in chimeras in spite of normal numbers of circulating T (E-rosetting) cells. The T_M-decrease/T_G-increase correlates with the appearence of GVHD. Equally significant depression of PWM-induced B-cell differentiation was found, and could not be rescued by depletion of suppressor (T_G)cells. Patients with severe combined immunodeficiency (SCID) also show T_M-depression/T_G-increase/PWM-induced B-cell differentiation-impairment [7] suggesting that this particular setting may be relevant to the clinical condition of impaired immune competence. As to the question of whether such condition is produced by an excess of suppressor T cells or by a lack of helper T-cells it should be recalled that T_G(suppressor) cells do not circulate in the "activated form" (except in particular settings as in the case of the fetus [3]) [7] so that an increase in the number of circulating T_G-cells does not necessarily mean that active suppressor mechanisms are operating: indeed in all our chimeras T_G-depletion *did not* eliminate "suppressor" capacity of chimera lymphocytes on control-B-cells, as would occur if T_G-cells were activated in vivo [7]. It is therefore suggested that helper activity is deficient in chimeras and may account for impaired immunoglobulin-synthesis, as already suggested by Gale and coworkers [1].

The fact that one patient had normal T_M-T_G levels until day+120, (Fig. 1) with no signs of GVHD, with normal haemopoiesis would suggest that "tolerance" versus host antigens may be achieved in the absence of T_M-T_G imbalancement: the same patient developed the usual setting i.e. T_M-depression/T_G-increase/PWM-induced differentiation-impairment, after clinical signs of chronic skin GVHD had appeared. More patients with no signs of GVHD have to be studied in order to ascertain the relevance of T_M-T_G imbalance in GVHD, in the process of tolerogenesis and in the post-transplant immunodeficiency.

Acknowledgements

This work was supported by C.N.R.-Rome CT 78.02845.96 and CT 79/78.02850.96/115.11801.

References

1. Gale, R. P., Opelz, G., Mickey, M. R., Graze, P. R., Saxon, A.: Immunodeficiency following allogeneic bone marrow transplantation. Transplant. Proc. *1*, 223–228 (1978)
2. Neely, J. E., Neely, A. N., Hersey, J. H.: Immunodeficiency following bone marrow transplantation: in vitro studies. Transplant. Proc. *1*, 229–232 (1978)
3. Hayward, A. R., Lawton, A. R.: Induction of plasma cell differentiation of human fetal lymphocytes: Evidence for functional immaturity of T and B cells. J. Immunol. *119*, 1213–1215 (1977)
4. Moretta, M. L., Ferrarini, M., Durante, M., Mingari, M. C.: Expression of a receptor for IgM by human T cells in vitro. Eur. J. Immunol. *5*, 565–567 (1965)
5. Moretta, L., Ferrarini, M., Mingari, M. C., Moretta, A., Webb, S.: Subpopulations of human T cells identified by receptors for immunoglobulins and mitogen responsiveness. J. Immunol. *117*, 2171–2174 (1976)
6. Moretta, L., Webb, S., Grossi, C. E., Lydyard, P. M., Cooper, M. D.: Functional analysis of two human T-cell subpopulations: help and suppression of B cell responses by T cell bearing receptors for IgM(T_M) or IgG(T_G). J. Exp. Med. *146*, 184–188 (1977)

7. Moretta, L., Ferrarini, M., Cooper, M. D.: Characterization of human T-cell subpopulations as defined by specific receptors for immunoglobulins. Contemp. Top. Immunobiol. *8*, 19–53 (1978)
8. Witherspoon, R., Noel, D., Storb, R., Ochs, D., Thomas, E. D.: The effect of graft versus host disease on reconstitution of the immune system following marrow transplantation for aplastic anemia or leukemia. Transplant. Proc. *1*, 233–236 (1978)

Discussion

Riethmüller: You said that Tμ cells are 70% of the peripheral E-rosette positive cell population. I wonder why you keep your total E-rosettes constant. Do you see any conversion of Tμ to Tγ cells under your incubation conditions?
Bacigalupo: To the first question: when you have a drop in the Tμ and no increase in Tγ, you have an increase in T null cells which is the case of some patients with severe combined immunedeficiency. Some patients with SCID have the inversion of Tμ–Tγ levels. To the second question: the answer is no, in this particular setting.
Dupont: Do you imply that the T subsets Tμ, Tγ corresponds in a way to what in the mouse is known as Ly-1, 2, 3 cells as suppressor and helper cell? Also I understand that there can be a transition from the Tμ to the Tγ?
Bacigalupo: In this particular in vitro-pokeweed differentiation model these cells work as helper and suppressor. I am not saying that these cells are suppressor in a more general sense.

Cooperation Between Thymus and Transplanted Precursor Cells During Reconstitution of Immunodeficiencies with Bone Marrow or Fetal Liver Cells

J.-L. Touraine

A. Introduction

The role of the thymus in the immunological reconstitution following transplantation of bone marrow or spleen cells in irradiated mice has been unequivocally established. The development of bone marrow transplantation in man for the treatment of severe combined immunodeficiency diseases (SCID), aplastic anemias and leukemias, over the last two decades has also demonstrated the importance of normal thymic function for complete immune reconstitution. The transplant of even less mature precursor cells, such as those contained in the fetal liver, leads to a slower T-cell maturation and the role of the thymus is still more crucial in such circumstances. It can thus be noted that combined thymus and liver cells from the same fetus are more efficient in reconstituting immunodeficient children than mere fetal liver cells. Several questions, of obvious practical significance, remain, however, unresolved: Are thymic epithelial cells necessary or can thymic factors replace the thymus organ? What are the thymic lesions in the diseases treated with marrow or fetal liver transplantation and are those lesions rapidly reversible? At which differentiation stages is the thymus more necessary? When the thymus expresses HLA antigens different from those of the lymphoid cells, does it induce an "allogeneic restriction" specific of the thymus type, will T cell functions be limited in a HLA-different host and will T cells be unable to cooperate with different B cells?

B. Patients, Materials and Methods

Eight patients with SCID have been treated: four of them received a bone marrow transplant and the other four received a fetal liver and thymus transplant. The eight patients had very typical, though varied, forms of SCID. They all had lymphopenia and decreased percentages of T-cells with surface differentiation antigens ($HTLA^+$) and forming E-rosettes. No in vitro proliferative response to PHA or Con A could be demonstrated and the allogeneic response was weak or absent. The numbers of immunoglobulin-bearing B-cells were variable but there were no plasma cells and virtually no identifiable antibody synthesis in any of the patients, although serum IgM levels were relatively high in some of them. Two patients had a genetic deficiency in adenosine deaminase (ADA) activity, the others did not. Each of the four infants treated with bone marrow transplantation had an HLA A,B and D identical sibling. Donor and recipient were of opposite sex in 3 cases. ABO incompatibility was present in 1 instance. Bone marrow

transplantation consisted of intravenous infusion of unfractionated 1.5×10^8 marrow nucleated cells per Kg of body weight (Touraine et al., 1978b). Each of the four patients treated with fetal liver and thymus transplants (FLTT) received a peritoneal injection of all liver and thymus cells obtained from a 8–13 week – old fetus. No attempt at HLA matching of the fetal donor and the recipient was performed.

One patient with a Di George syndrome, characterized by a significant hypoparathyroidism and decreased T cell numbers and functions, was treated with a fetal thymus transplant.

The in vitro induction assay was performed using human bone marrow cells separated into five layers by centrifugation on a discontinuous bovine serum albumin density gradient (Touraine et al., 1974). Marrow cells from the various layers were incubated for 2 to 18 hours with thymic factors, then assayed for surface characteristics of T lymphocytes. Lymphocyte cultures, in the presence of phytomitogens or mitomycin-treated allogeneic lymphocytes, were carried out in microtiter plates.

C. Results and Discussion

I. Sequential T-Lymphocyte Differentiation Under Thymic Influence

In previous reports we have shown that certain cells from human bone marrow can acquire T-cell characteristics (including the $HTLA^+$ phenotype and the capacity to form-E-rosettes) after incubation with thymic factors (Touraine et al., 1974; Touraine et al., 1977). The sequential differentiation has been demonstrated to involve firstly some differentiation antigens, then the membrane characteristics leading to E-rosette formation and later the properties to proliferate in response to mitogens or allogeneic stimuli (Touraine et al., 1977). More recent experiments have enabled us to further obtain precisions on several of the T-cell subpopulations which develop under the influence of the thymus (Fig. 1). Some T-cell subsets can respond to allogeneic cells by proliferation and/or by generation of cytotoxic cells. Helper cells may amplify the cytotoxic response. Other T-cell subsets acquire the proliferative response to mitogens (Con A, PHA) and some of them can exert a suppressive function on allogeneic-responsive cells. Still other T-cells respond to different mitogens such as phorbol myristate acetate (PMA).

Virtually every stage of T-cell differentiation has been shown to be enhanced by in vitro incubation with thymic factors. Such factors, as they are presently available, cannot however induce the full development from stem cells to mature T lymphocytes.

In the induction assay, patients with SCID appear to have no, few, or abnormal inducible T-cell precursors in their bone marrow, in contrast with all other donors tested, including patients with the Di George syndrome and several patients with "pure" T-cell deficienccies. Following marrow or fetal liver transplantation, however, SCID patients readily develop normal numbers of these inducible T-cell precursors from the transplanted cells.

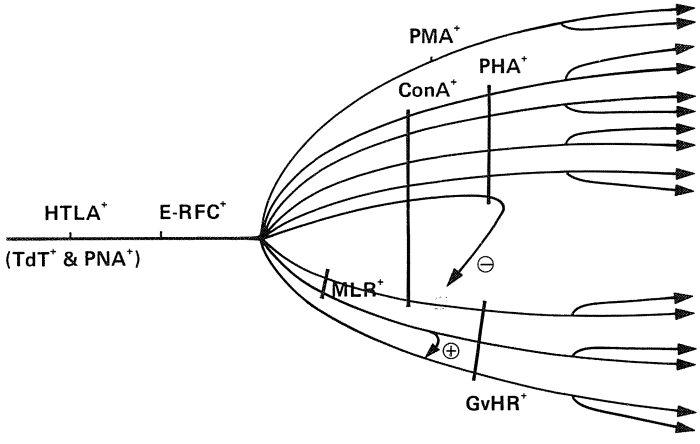


Fig. 1. Proposed scheme of T lymphocyte differentiation in man. HTLA = human T lymphocyte differentiation antigens; E-RFC = E-rosette-forming cells; PMA = phorbol myristate acetate; ConA = concanavalin A; PHA = phytohemagglutinin; MLR = mixed lymphocyte reaction; GvHR = graft-versus-host reaction

II. Fetal Thymus Transplantation in Di George Syndrome

Our attempts to treat patients with T cell deficiencies, including Di George syndromes, with thymic factors or even with thymic epithelial cells from prolonged cultures have, so far, resulted in only limited clinical benefit, despite increased T cell numbers and in vitro functions in several cases. In contrast with such mild alterations, a transplant of a fetal thymus in an infant with a Di George syndrome had a rapid and drastic result (Fig. 2). Full immunological reconstitution occurred, although HLA antigens of the transplanted thymic epithelial cells and of lymphoid precursors were different. All peripheral T lymphocytes developed from the recipient's own precursors differentiating under the influence

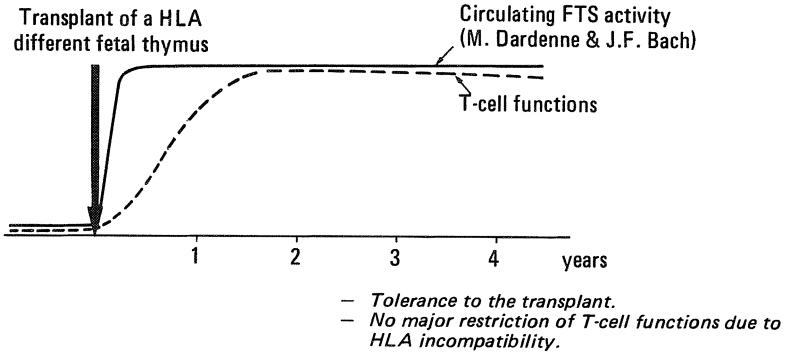


Fig. 2. Follow-up of a patient with Di George syndrome over four years following a fetal thymus transplant

of the donor's thymic epithelium, as shown by chromosomal analysis. The circulating thymic factor (FTS) activity in serum, repeatedly determined by Drs M. Dardenne and J. F. Bach, was extremely low before the transplant, increased rapidly thereafter and has remained normal, suggesting tolerance to the graft, as also observed in a related model in the mouse (Kindred and Loor, 1974). Furthermore the "restriction" of some T-cell responses by the major histocompatibility complex (MHC) of the thymic epithelium, demonstrated to occur in mouse experiments (Zinkernagel, 1978), did not appear to significantly limit the reconstitution of T cell functions in this patient.

III. Bone Marrow Transplantation in SCID

The SCID syndrome is a relatively heterogeneous group of diseases. Careful analysis of thymus morphology in such patients has revealed five different patterns: "total dysplasia, partial dysplasia, heterogeneous cell population, partial dysplasia with phagocytosis and late fetal pattern" (Borzy et al., 1979). Patients with all varieties of SCID can however be cured by the transplant of a compatible bone marrow. As shown in Table 1, 3 of the 4 patients that we treated with marrow transplantation were immunologically reconstituted and are now alive, in perfect condition, 4 years later. The fourth patient died of a previously generalized BCG infection. Following the transplants, evidence of engraftment was obtained (XX chromosomes in male recipients, Gm immunoglobulin groups). In at least one patient T-cells derived from the donor cells while B-cells remained of recipient origin but this chimerism did not result in any difficulty in T-B-cell cooperation.

In another child treated with bone marrow transplantation for aplastic anemia, a more complex chimerism was demonstrated. After appropriate in vitro stimulation with mitogens, T and B lymphoblasts were separated using specific anti-T and anti-B heteroantisera. The T cell population could then be shown to include a majority of cells of donor origin (XX) and some cells of recipient origin (XY). Conversely B cells mostly derived from recipient cells and a small proportion from the donor cells.

In all circumstances, the part played by the thymus in T-cell development is crucial. T lymphocyte differentiation in vivo involved the same stages as demonstrated in the induction experiments in vitro. Relatively long periods of time were, however, necessary for full-T-cell development in vivo where some association – or succession – existed between differentiation and proliferation processes.

A delayed reconstitution of full T-cell immunity may be explained by thymic abnormalities in SCID (Borzy et al., 1979) as well as in marrow transplanted patients with aplastic anemia or acute leukemia (Beschorner et al., 1978).

IV. Fetal Liver and Thymus Transplantation in SCID

As summarized in Table 1, 3 of the 4 patients treated by FLTT, because they lacked a compatible marrow donor, are still alive. Two patients are at an initial

Table 1. Bone marrow and fetal tissue transplantation in severe combined immunodeficiencies

Patient	Treatment	Take	GvHR	Reconstitution	Outcome	Follow-up
T.♂[a]	BMT (sister)×2	+	+	Full	Perfect	4 years
C.♀	BMT (sister)×2	+	−	Almost full	Perfect	4 years
M.♂	BMT (sister)	+	−	Full	Perfect	4 years
H.♀	BMT (brother)×2	+	−	Beginning only	Died of previous BCG infection	1 month
S.♂	FLTT×2	+	−	Almost full	Perfect	2 years
B.♂[a]	FLTT	?	−	No	Died of previous BCG infection	1 month
C.♀	FLTT×2 (+CETT)	?	−	Initial	Satisfactory in sterile isolation	1 year
F.♀	FLTT×2	?	−	Initial	Satisfactory in sterile isolation	1 year

[a] Patients with adenosine deaminase deficiency
BMT=bone marrow transplant; FLTT=fetal liver and thymus transplant; CETT=cultured epithelial thymus transplants

stage of reconstitution and one now has a virtually complete immunological reconstitution. The addition of the thymus from the same fetus as the liver seems to increase the probability of a successful and uneventful transplantation (Pahwa et al., 1977). It enhances the maturation of T lymphocytes from the immature fetal liver cells, it may provide better conditions for this differentiation and it has been said to lower the incidence of the graft-versus-host-reaction (GvHR).

In the child with a 28 month follow-up, a slow but complete T cell development has been observed (Fig. 3). No GvHR developed. By HLA typing of separated T and B lymphocytes, is was possible to demonstrate chimerism among lymphocyte populations, all T lymphocytes deriving from the donor's precursor cells and all B lymphocytes from the recipient's. Cooperation between such T and B cells of completely different HLA phenotypes seemed to be possible in vivo and it resulted in the appearance of a normal antibody production. The avidity of antibodies has not yet been tested and it may be relatively low, explaining a few infectious episodes with extracellular organisms. In vivo defense mechanisms against viruses have been normal and preliminary data suggest that T cell functions may not be severely restricted by the MHC incompatibility. It is difficult to ascertain whether most of the T lymphocytes, which derived from the donor cells, differentiated in the environment of the grafted thymic epithelial cells (with identical HLA phenotype) or of the recipient thymus (with different HLA phenotype). Whatever the case, the T lymphocytes, expressing HLA antigens different from HLA antigens of B cells and of other host cells, were capable of at least some degree of activity on those cells (Fig. 4).

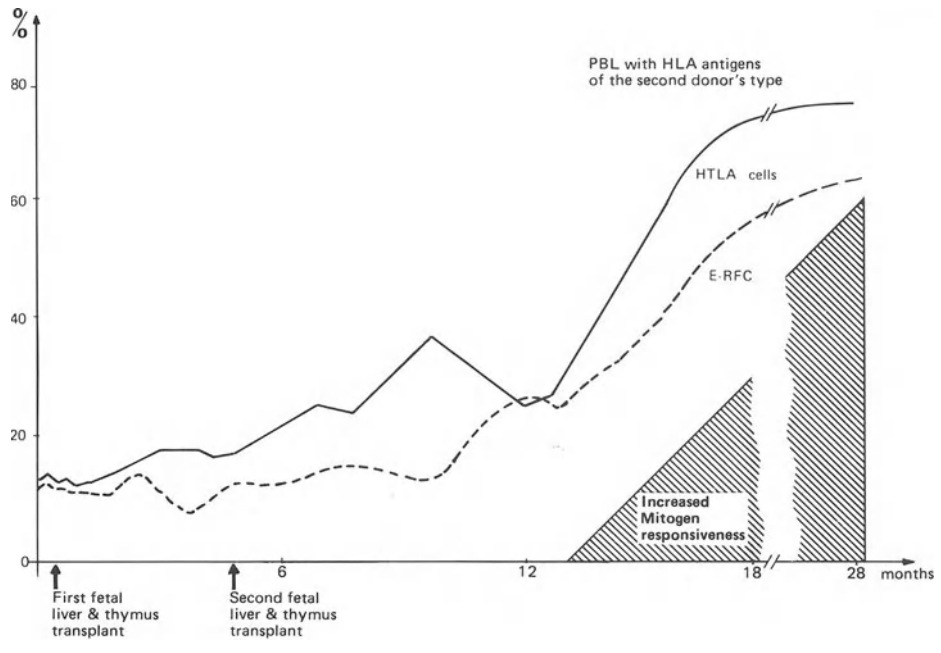
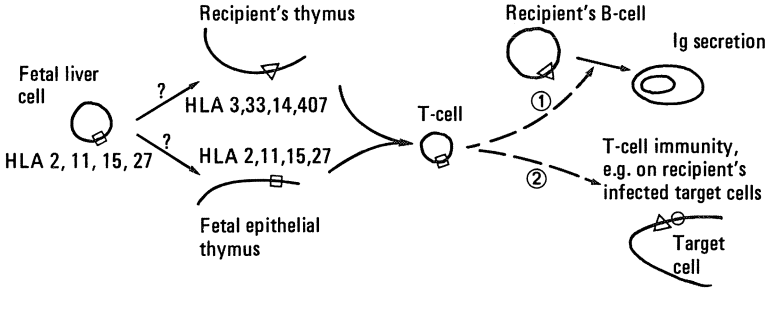


Fig. 3. Progressive T-cell reconstitution of a patient with severe combined immunodeficiency disease following fetal liver and thymus transplants



① Some degree of T-B-cell cooperation across MHC barrier

② No absolute MHC restriction of T-cell functions

Fig. 4. Scheme of the immunological reconstitution of a patient with severe combined immunodeficiency disease following fetal liver and thymus transplants. T lymphocytes derived from the donor precursor cells and differentiated under the influence of either the donor's or the recipient's thymus. In contrast, B lymphocytes as well as other host cells express HLA antigens different from those of T lymphocytes

D. Conclusion

Although thymic factors are potent inducers of T-cell differentiation of already committed prothymocytes, interaction with the thymic epithelium itself is more efficient in vivo. Knowledge of the sequential development of T lymphocytes provides a useful means to follow immunological reconstitution following bone marrow or fetal tissue transplantation. The restriction by the MHC of T cell responses may not be absolute in human transplantation as thought from mouse experiments (Zinkernagel, 1978). Several explanations for a less significant "restriction" can be hypothesized and may be further analyzed in animal experiments: a) partial in vivo restriction when more absolute restriction appears to exist in vitro by lack of very discriminative assay; b) absolute restriction in short experiments as in the mouse, but a partial one in long-term T-cell reconstitution; c) restriction partially imposed by the thymus MHC, partially by the lymphoid cell MHC; d) absolute restriction in inbred animals, but a partial one in outbred individuals in which many cross-reactions between MHC determinantes have been demonstrated; e) possibility to circumvent the restriction, e.g. by progressive development of allo+X recognition in the place of self+X recognition.

These studies have already confirmed to us the necessary interactions between the thymus and the precursor cells. Continued investigations in such informative cases will give answers to the still unresolved questions concerning the subtle mechanisms by which the thymus-derived lymphocytes develop their recognition structures and differentiate into several subsets with distinct functions. MHC determinants play a part in such processes, as also suggested in man by the association of immunodeficiency and lack of expression of HLA antigens (Touraine et al., 1978b). The precise role of these MHC determinants as well as of surface differentiation antigens needs, however, further analysis.

Acknowledgements

This work was supported by grants from the "Centre National de la Recherche Scientifique", the "Institut National de la Santé et de la Recherche Médicale" and the "Fondation de France".

References

Beschorner, W. E., Hutchins, G. M., Elfenbein, G. J., Santos, G. W.: The thymus in patients with allogeneic bone marrow transplants. Am. J. Pathol. *92*, 173–186 (1978)

Borzy, M. S., Schulte-Wissermann, H., Gilbert, E., Horowitz, S. D., Pellett, J., Hong, R.: Thymic morphology in immunodeficiency diseases: Results of thymic biopsies. Clin. Immunol. Immunopathol. *12*, 31–51 (1979)

Kindred, B., Loor, F.: Activity of host-derived T cells which differentiate in nude mice grafted with co-isogenic or allogenic thymuses. J. Exp. Med. *139*, 1215–1227 (1974)

Pahwa, R., Pahwa, S., Good, R. A., Incefy, G. S., O'Reilly, R. J.: Rationale for combined use of fetal liver and thymus for immunological reconstitution in patients with variants of severe combined immunodeficiency. Proc. Natl. Acad. SCI. USA, *74*, 3002–3005 (1977)

Touraine, J. L., Bétuel, H., Souillet, G., Jeune, M.: Combined immunodeficiency disease associated with absence of cell-surface HLA-A and -B antigens. J. Pediat. *93*, 47–51 (1978a)
Touraine, J. L., Freycon, F., Chataing, B., Souillet, G., Salle, B., Philippe, N., Bétuel, H., Lauras, B., Touraine, F., Jeune, M., Monnet, P.: Experience with bone marrow transplantation for severe combined immunodeficiencies in Lyon and Saint-Etienne. Pathol. Biol. (Paris) *26*, 27–28 (1978b)
Touraine, J. L., Hadden, J. W., Good, R. A.: Sequential stages of human T lymphocyte differentiation. Proc. Natl. Acad. Sci. USA *74*, 3414–3418 (1977)
Touraine, J. L., Incefy, G. S., Touraine, F., Rho, Y. M., Good, R. A.: Differentiation of human bone marrow cells into T lymphocytes by in vitro incubation with thymic extracts. Clin. Exp. Immunol. *17*, 151–158 (1974)
Zinkernagel, R. M.: The thymus: Its influence on recognition of "self major histocompatibility antigens" by T cells and consequences for reconstitution of immunodeficiency. Springer Semin. Immunopathol. *1*, 405–415 (1978)

Discussion

Kersey: I have the impression that the lymphocytes of your patient with the bare lymphocyte syndrome did not differentiate "in vitro" under thymic influence. Do you think that HLA antigens are necessary for differentiation or is there another defect in the patient's lymphocytes?
Touraine: There are several possible explanations, one of which is indeed that the phenotypic expression of HLA or β_2-microglobulin on the cell surface is needed for lymphocyte differentiation. It may also betressed that we could demonstrate HLA antigens in the serum. So, there is not a defect in HLA genotype.
Niethammer: Did the patient in whom you had slow reconstitution of T cells produce specific antibodies and when did he start to make these?
Touraine: He produced specific antibodies starting more than one year after grafting. Their levels are not yet normal, but slowly increasing.
Dupont: We have similar experience with fetal liver and thymus transplants with good T- B-cell cooperation, but this patient lost the graft after more than one year and is now back with severe combined immune deficiency. So this tolerance can be broken rather late. How many patients live after more than two years?
Touraine: I agree, this case and maybe another one are the only two I know of.
van Bekkum: You have given fetal liver cells and thymus of the same fetus i.p. Being critical I would think that the fetal liver cells are educated in the recipient's thymus, since this has been seen with marrow grafts and this route appears rather ineffective for epithelial thymus grafts.
Touraine: In general, I would agree. We don't know whether reconstitution developed under the influence of one or the other fetal thymus.
Waldmann: As one who believes in MHC restriction of T-B-cell cooperation I could think not only of the thymic epithelium confering restriction, but also of some long lived radiation resistant cell in it.
Touraine: Contrary to mouse experiments, in the human situation we have long term observations and the MHC restriction may hypothetically be circumvented after prolonged time by development of alternative recognition structures.
Kay: Is the split chimerism in your patients related to the type of SCID, ADA deficiency of other? Secondly, do you treat your patients with ADA deficiency first with red cell transfusions and if these fail with fetal liver transplants?
Touraine: It depends on the type of SCID and the type of treatment. In patients with B-cells, there is a deficiency of T-cell precursors and only T-cells engraft wich cooperate with remaining host B-cells. In cases without any B cells, one can get both T and B cell replacements or only T cell replacement in which case a B cell defect remains. This is especially so for reconstitution with fetal liver which seems to replace T more than B cells. In a case of bone marrow transplantation for aplastic anemia, we saw partial chimerism in T and B cells. In most cases of split chimerism the T cells are predominantly of

donor and the B cells of host origin. With regard to red cell transfusions in ADA deficiency I have to say that we have not tried in our two cases, because one had an HLA identical sibling as marrow donor and is completely reconstituted, the second died of a generalized BCG infection. I would only propose to give red cells as a first trial in cases without HLA identical donor.

Thiel: You pointed out that a T antigen positive T cell without E receptor precedes a cell carrying both. Did you find in your patients before and after reconstitution T antigen positive cells without E receptors?

Touraine: Yes, we found T cell deficient patients with HTLA positive cells unable to bind sheep red cells and found during reconstitution of our patients a time lasting days to weeks, when they had HTLA positive cells without E receptor. However, we know that both markers are indipendent from each other and it is conceivable that in some cases E receptors can develop in the absence of a large density of HTLA at the all surface.

Dupont: In our experience, these patients which you describe as having B cells also have some ability to react in MLR, but not to PHA. These are just incomplete SCID patients. The split chimerism is related to the fact that we do not pretreat these patients what we might do in the future, expecially in patients with incomplete SCID.

Touraine: Pretreatment may be of value for mismatched grafts. I agree that it is somewhat schematical to speak about SCID patients with and without B cells, since some have a low T cell function left and a variety of other differences may exist in this relatively heterogeneous group of diseases.

4 Suppression of Graft Versus Host Reactions with Antisera Against Cell Surface Markers

Restitution Potentials of Allogeneically or Xenogeneically Grafted Lymphocyte-free Hemopoietic Stem Cells

W. Müller-Ruchholtz, H.-U. Wottge, and H. K. Müller-Hermelink

A. Introduction

I. Aims

Bone marrow transplantation (BMT) has attracted increasing interest from both clinical as well as basic research oriented viewpoints, because manipulation of hemopoietic stem cells offers a promising approach to a remarkable variety of problems. The clinician, obviously, is interested in the replacement of hemopoietic tissue which may be lacking because of some "genuine" defect, some sort of accident or the intentional strengthening of cancer therapy. (This aspect is broadly covered in other papers of this series.) The basic immunologist may be attracted because he can provide himself with a tool for functional as well as morphological studies to better understand the maturation of immune reactivity. And finally, clinicians and research workers will be highly interested in the elaboration of means to manipulate immune reactivity by manipulating BM immune reactivity when considering the fact that the hemopoietic stem cell continuously gives rise to lymphocytically differentiating progeny at any age of life, early in ontogeny as well as late in the adult organism.

II. Problems

On the other hand, it is the immune reactivity potential, already existing at a given moment and represented by the less or more diferentiated BM lymphocytes, which leads to the well-known main problems of BMT unless the graft is autologous or syngeneic. The underlying graft versus host reactions (GVHR) have been known since Simonsen's early description and discussion far more than 20 years ago, and have been covered in excellent reviews (e.g. Simonsen, 1962; Elkins, 1971); thus there is no need for further explanation here.

A second major problem may be seen in the foreign environment to which the grafted hemopoietic cells are exposed, increasing with the dimensions of the so-called histoincompatibility barrier (less prejudically called histodifference) between donor and recipient. Even when eliminating immunological host versus graft reactions, by using parent/F_1 hybrid combinations or recipient pre-irradiation etc., genetically determined restrictions of engraftment or graft function have been described and/or discussed (see Elkins, 1979; Zinkernagel, 1978).

III. Variety of Approaches to Lymphocyte Inactivation

To eliminate or at least inactivate the lymphocytes present in BM, has been and still appears to offer the best potential for success in BMT. Indeed, activities along this line allowed better identification of the second group of problems mentioned above and there is reason to expect that they will also help to solve them.

The large variety of approaches which has been tried over the past 15 years, it outlined in Fig 1. However, it is not intended to add examples of the one or the other procedure; the reader of this series of papers will easily find for himself where to group the present activities.

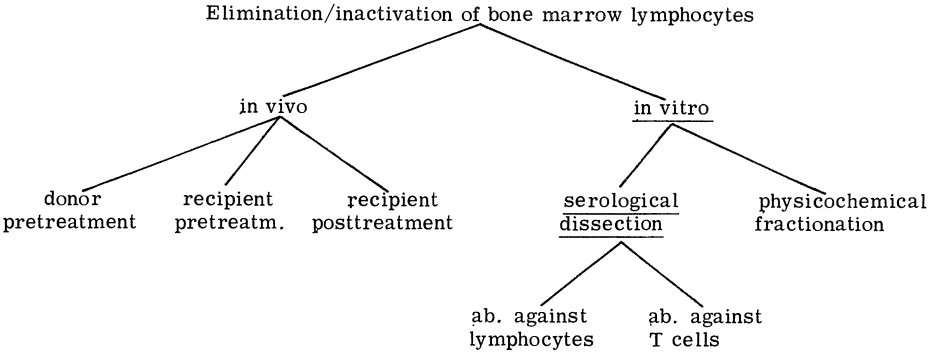


Fig. 1. Survey of approaches

B. Own Approach

I. Principle

We have chosen the in vitro treatment of BM in order to avoid the disadvantages and restrictions of treating the donor or recipient; and we have concentrated on what has been termed "serological dissection", assuming that antibodies against antigenic determinants on lymphocytes not shared by hemopoietic stem cells represent the best dissecting tool.

Figure 2 illustrates this approach. The in vitro incubation of adult donor BM with antilymphocyte serum lasts only 40–45 min. The objective is the study of the morphological and functional development of lymphocytes newly differentiating from the grafted stem cells.

II. Methodology

The experiments were performed in rat → rat and in rat → mouse models. A large part of methodological details has been described previously (Müller-Ruchholtz et al., 1975, 1976), some will be given in the result chapters. In brief, the following procedures were used:

Antisera: Rabbit-anti-rat lymphocyte-sera (ALS-O) were produced by i.v. injections of 5×10^8 thymocytes (thymuslymphocytes) twice weekly for 3 weeks. ALS-E was prepared by absorption with rat erythrocytes, 3× with equal volumes, ALS-EP by 3 subsequent absorptions with 2×10^8/ml cultivated lymphocyte-free peritoneal exudate cells, ALS-EPLf by 3 additional similar absorptions with fetal liver cells and ALS-EPLfBl similarly by further absorptions with B-rat lymphocytes (spleen cells obtained 6 weeks after restitution of rats that were thymectomized, lethally irradiated and restituted with syngeneic BM). ALS-EPLf will be referred to as SAL, specified anti-lymphocytic serum, since it could be shown (see also below) that it completely lacked anti-hemopoietic stem cell

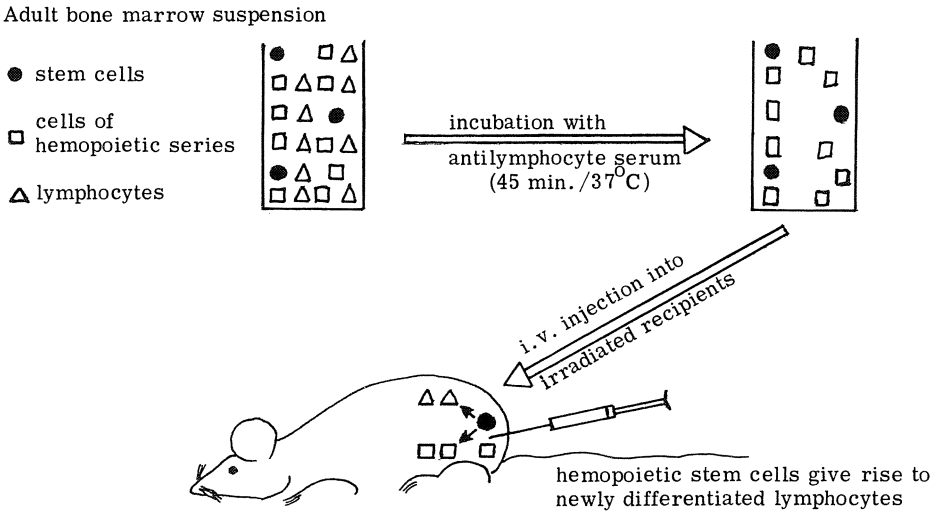


Fig. 2. Principle of own approach

activity maintaining anti-prethymic precursor activity. ALS-EPLfBl will be referred to as SAT, specific anti-thymocytic serum, since its activity was further restricted to thymocytes and postthymic cells.

Animals: Lewis rats (LEW), 3–4 month old females lethally irradiated with a ^{60}Co source,were used as BM recipients. Young adult CAP rats served as donors in allogeneic BMT experiments, i.e. fully (not semi-) allogeneic, MHC-plus non-MHC-different inbred strain combinations were chosen (RT 1^c → RT 1^l). They further served as donors in xenogeneic BMT experiments with lethally irradiated BALB/c or C3H mice as recipients.

C. Absorption Analysis of Relevant Thymocyte Surface Antigens

The native ALS-O may be defined as an antiserum raised in another species by immunization with thymus lymphocytes and containing various antibodies against a variety of lymphoid cell surface antigens, most of which are shared by other cells. Therefore a set of absorptions with cross-reacting cells was required to increase the ALS specificity in a defined way in order to make it useful for the approach outlined above. The various absorption experiments allowed some insight into the pattern of sharing of cell surface structures between related cells, and the data presented here also allow one to understand why a certain set of absorptions has been considered appropriate for the further BMT incubation experiments.

I. Serological Evaluations

1. Lymphocytotoxicity

Thymocytotoxic titers dropped in ALS-O → ALS-EPLf (=SAL) from 1:256 – 1:512 → 1:32 – 1:64. This titer drop occurred stepwise only when consecutive

absorptions with different cross-reactive cells were performed, and not upon further repeated absorptions with the same cells. This indicates 1. that each step of absorption was performed exhaustively and 2. that there remained a reasonable lymphocytotoxic titer in the SAL.

Comparing the lymphocytotoxicity of SAL and SAT it could be shown 1. that in the latter thymocytotoxic titers had dropped by a factor of 8, and 2. that SAT reacted only against a subpopulation of spleen or lymph node lymphocytes, in contrast to SAL, as indicated in Fig. 3.

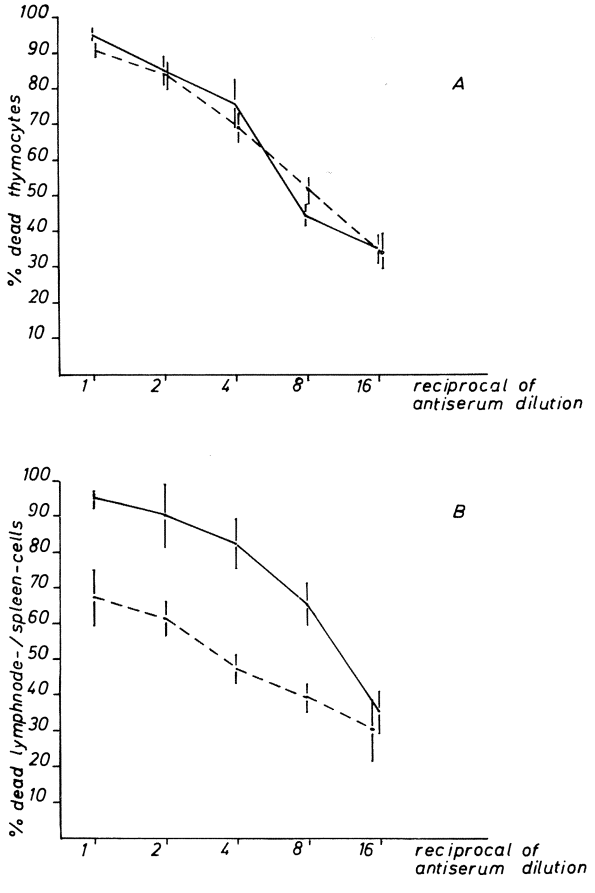


Fig. 3. Lymphocytotoxic activities of SAL (——) and SAT (– – –) against thymocytes *(A)* and lymph node/spleen cells *(B)*. To allow comparison in *(B)* at equithymocytotoxic levels, SAL was prediluted 1:8. The rat cells were incubated with antisera for 30 min., then undiluted fresh normal rat serum was added as the source of complement; 120 min. later the number of living (!) cells was counted in dye exclusion tests and expressed as percentage of living cells in control incubations with normal serum. N=6 for each cell/antiserum combination

2. BM Cytotoxicity

The unabsorbed ALS-O killed more than 90% of nucleated BM cells when tested in dye exclusion tests, and practically all cells when tested cytologically on stained smears. As to be expected and as seen in Fig. 4, this percentage dropped with progressive absorptions down to ~22% in ALS-EPLf. The latter value was significantly ~12–14% above background and in the order of the lymphocyte percentage in rat BM. However, as also indicated in Fig. 4, similar quantitative alterations could also be obtained by simple dilutions, i.e. titer reductions, of the

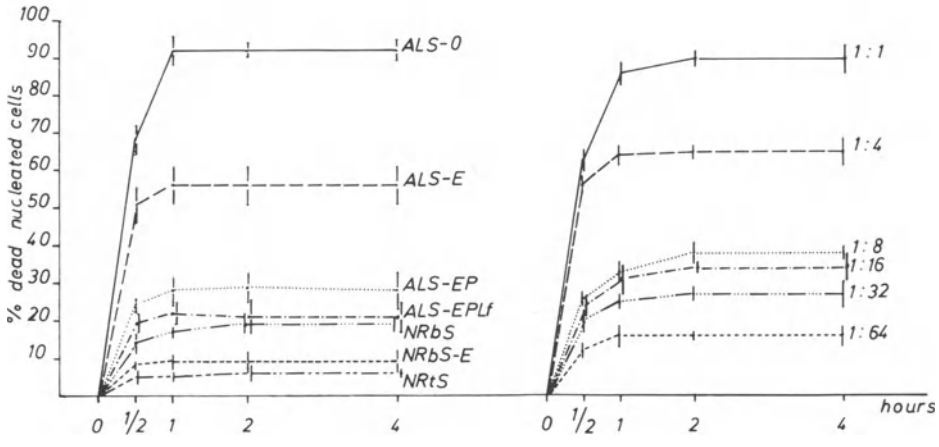


Fig. 4. Cytotoxic activity of ALS against nucleated rat bone marrow cells. Quantitative comparison of the effects of the same antiserum when used undiluted but progressively absorbed or unabsorbed but progressively diluted. Fresh rat serum served as the source of complement. Evaluation with trypan blue exclusion tests. Each dot represent the data from 8 experiments

unabsorbed ALS-O. Therefore, only additional qualitative studies would allow to state a real specificity increase by the absorptions.

II. Myelogram Evaluations

In order to overcome these limitations myelograms were prepared to compare the differential counts of myelopoietic cells, erythropoietic cells and lymphocytes in the absorption series and the dilution series at several equicytotoxic levels. Fig. 5 shows the results. ALS-EPLf still destroyed practically all lymphocytes while myelopoietic and erythropoietic cells appeared intact again at the normal ratio of 7:2. However, diluted ALS-O left more and more lymphocytes intact. These data indicate a real increase of ALS specificity due to the absorptions rather than a spurious one caused by reduction of the titer.

III. Electronmicroscopic Evaluation

The validity of the observed specificity increase due to the described absorptions was further supported by the following experiments: BM cells were incubated in ALS without complement, 4–5× washed and then incubated with peroxidase-conjugated sheep-antirabbit IgG. The immunperoxidase labeling of cell membranes, published in detail elsewhere, is here summarized in Table 1. After incubation in ALS-O, the surface of all cells showed a continuous layer of peroxidase reactions products. Absorption with erythrocytes removed mainly antibodies cross-reactive with late erythropoietic cells. Additional absorptions with peritoneal cells removed other antibodies, cross-reactive with late myelopoietic cells, megakaryocytes and with immature myelopoietic and erythropoietic cells (leaving, however, a characteristic, distinct patchlike labeling of the latter).

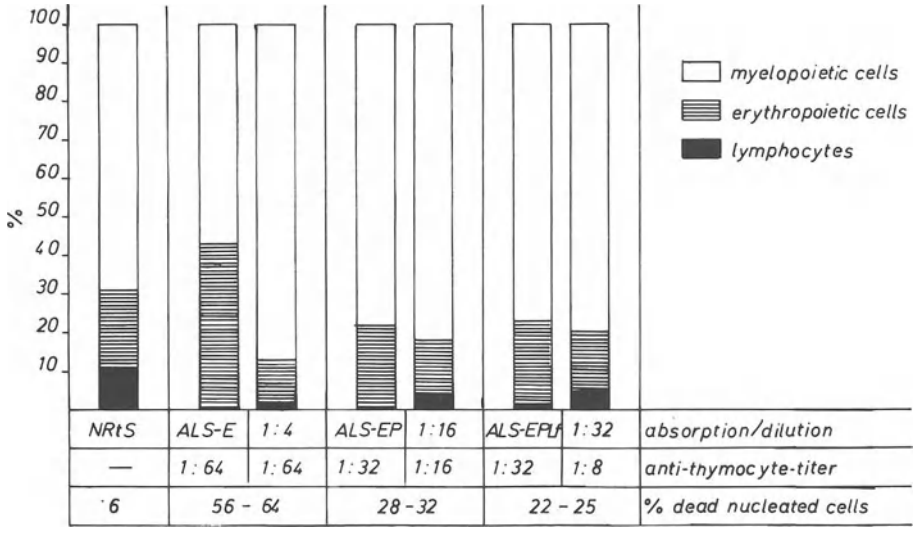


Fig. 5. Cytotoxic activity of ALS against nucleated rat bone marrow cells. Qualitative comparison of the myelograms obtained with undiluted but progressively absorbed or unabsorbed, progressively diluted antiserum at equicytotoxic levels. Differential counts of 1000 bone marrow cells per smear after 2 hrs. of incubation with the antiserum (see Fig. 4)

Table 1. Survey of electron microscopic evaluations of rat bone marrow cells following incubation with progressively absorbed ALS and subsequent immunoperoxidase labeling. (+) indicates clearly positive but only patchlike labeling, whereas + indicates continuous cell surface labeling

Cell type		Absorption level of the ALS				Control sera (NRtS, NRbS-EP)
		Unabs.	ALS-E	ALS-EP	ALS-EPLf	
Lymphocytes		+	+	+	+	−
Myelopoietic cells	< early	+	+	(+)	−	−
	< late	+	+	−	−	−
Erythropoietic cells	< early	+	+	(+)	−	−
	< late	+	−	−	−	−
Megakaryocytes		+	(+)	−	−	−

Subsequent absorption with fetal liver cells removed a further group of antibodies, cross-reactive only with these immature hemopoietic cells. ALS-EPLf led, however, still to a continuous surface labeling of lymphocytes, lymphoblasts and plasma cells.

IV. Analysis Summary

Taken together the various absorption studies allow one to postulate at least 6 types of thymocyte surface antigens with varying patterns of expression on other cells. This is shown in Table 2. "Type" does not necessarily mean a single

Type (No.)	Pattern of sharing with other cells
I	All cells including erythrocytes
II	BM lymphocytes, granulocytes, macrophages, myelopoietic and erythropoietic cells, BM stem cells
III	BM lymphocytes, erythropoietic cells, fetal liver and BM stem cells
IV	BM lymphocytes, myelopoietic cells, fetal liver and BM stem cells
V	BM lymphocytes
VI	Thymocytes, but no other lymphocytes

Table 2. Types of thymocyte surface antigens. Incomplete data, restricted by evaluation only in relation to bone marrow cells and detection capacity of rabbit antirat ALS

specificity because there may by several with similar patterns of distribution among the cells investigated. Though far from complete, these data indicate the network of structural relationship between related cells which should be considered in order to prepare from ALS an SAL or SAT.

D. In Vivo Characterization of SAL Specificity

The findings described in this chapter were performed in syngeneic BMT experiments in order to obtain hematological informations without possible interference by transplantation-immunological reactions.

I. Evidence for Activity Against Prethymic Precursors

The restitution of thymus was studied at weekly intervals, comparing lethally irradiated recipients of non-incubated BM with recipients of SAL-incubated BM. Gross morphology revealed that the thymus size and weight recovery plateau was reached after about 3 weeks in the former groups and after about 6 weeks in the latter. This delay of thymus recovery by 3 weeks in the SAL-incubated BM group was strikingly confirmed with histological evaluations, as presented in Fig. 6. Three weeks after grafting of fresh, untreated BM, the thymus shows again its typical lymphoid organization with the dark stained lymphocyte-rich cortex and the light stained medulla. At the same time, no lymphoid recolonization is seen after transplantation of SAL-incubated BM which, however, is complete at 6 weeks.

Additional support of the hypothesis that SAL eradicates lymphoid precursor cells may be taken from differential white blood cell counts of restituted animals. As shown in Fig. 7, after 3 weeks granulocytes as well as lymphocytes had reached normal levels in the recipients of untreated BM whereas this was the case only for granulocytes in the SAL-incubated BM group.

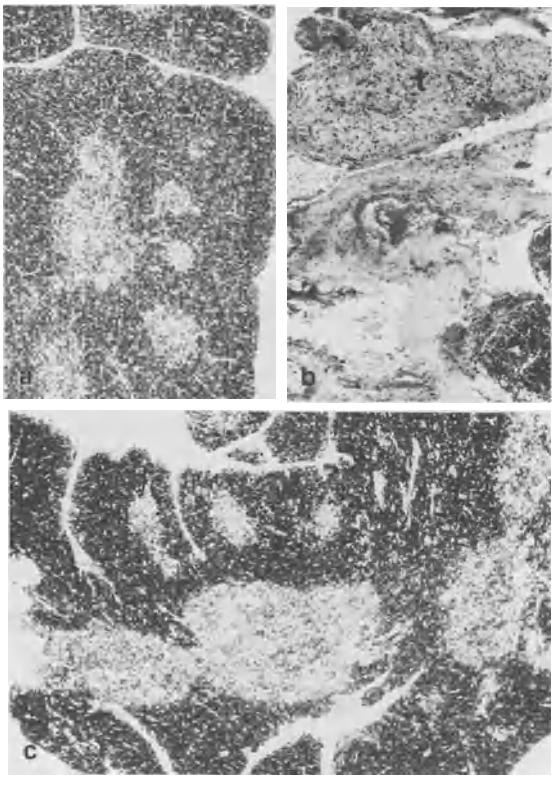


Fig. 6. Thymus morphology in rats after lethal irradiation and transplantation of syngeneic BM: **a** 3 weeks after grafting of untreated BM; **b–c** 3 weeks and 6 weeks after grafting of SAL-incubated BM. H & E; 64×; t = thymus epithelial remnant

II. Evidence for Lack of Activity Against Hemopoietic Cells

Bone marrow cell dose/response curves were established, measurring the survival rates of lethally irradiated recipients of BM treated in various ways. The antiserum-incubated cells were washed once and injected i.v., half of them 2 and 18 hrs after irradiation. As shown in Fig. 8, no significant differences were found between restitution capacity of untreated BM cells, those incubated in absorbed normal rabbit serum and those incubated in SAL. These data demonstrate functionally the absence of any cytotoxicity of SAL for hemopoietic cells.

On the other hand, ALS-EP still completely prevented restitution. Furthermore, not indicated in Fig. 8, ALS-ELf abolished the restitution potential of 2×10^8 BM cells in 8/8 experiments. These observations are in complete accordance with the cytological findings described in chapter C III and prove that combined absorptions were required for the preparation of an appropriately functioning SAL.

E. Restitution of Allografted BM Recipients

All experiments described in this chapter were done in a fully (not semi-) allogeneic MHC – plus non-MHC-different strain combination.

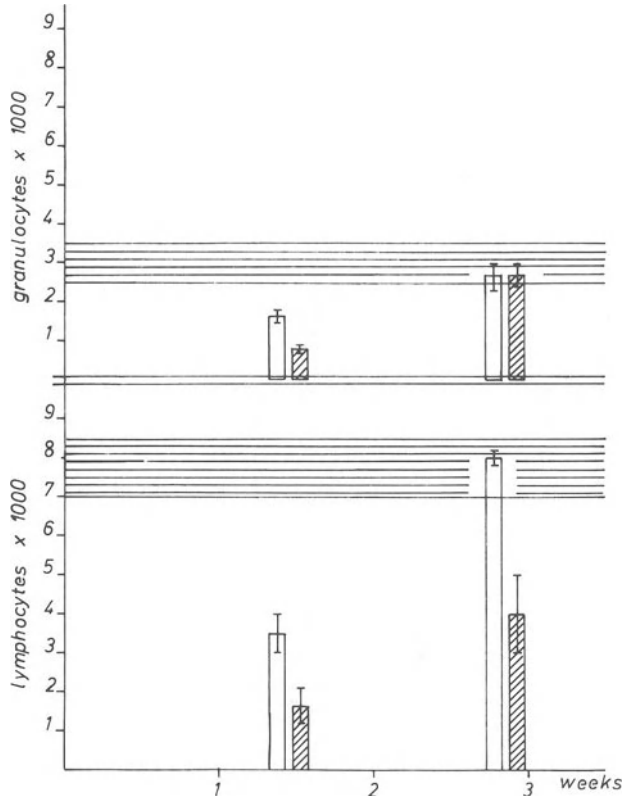


Fig. 7. White blood cell restitution following lethal irradiation and grafting of syngeneic bone marrow in LEW rats. Open columns: untreated BM; hatched columns: SAL-incubated BM; horizontal hatching: normal range; N for each experiment: 10

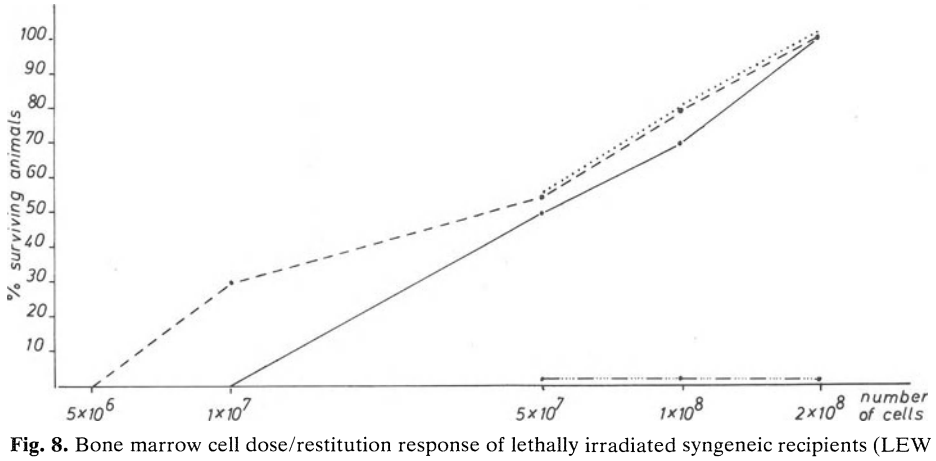


Fig. 8. Bone marrow cell dose/restitution response of lethally irradiated syngeneic recipients (LEW rats).
Comparison of the restitution capacity of untreated BM (– – –), normal rabbit serum (EP-absorbed) incubated BM (...), ALS-EP incubated BM (–...–) and ALS-EPLf (=SAL) incubated BM (——). N per cell dose and serum: 10–15

I. BM Pretreatment with SAL

1. Recipient Survival

The survival rates are presented in Fig. 9. Irradiation controls died after 9–20 days. Recipients of untreated BM survived only a few days longer. They developed an acute and fatal GVHR with characteristic alterations of thymus and lymph nodes (see below), lymphoid infiltrations of liver, skin and intestinal wall, lymphopenia (~60%), granulocytosis (~200%) and a typical sharp weight loss curve. In contrast, recipients of SAL-incubated BM mostly showed a delayed, but complete restitution. Only a small percentage died, merely within the 3rd and 4th week of the irradiation recovery phase, from infections due to conventional housing conditions. The great majority survived in good health for the full observation period (in 12 cases for more than 1 year).

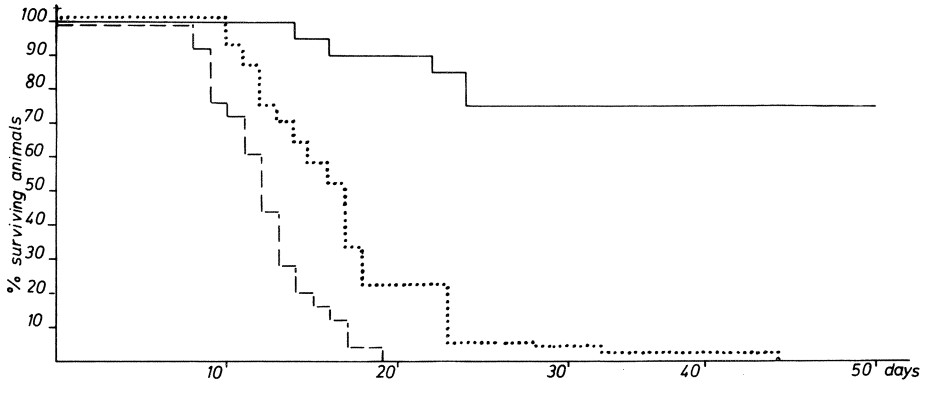


Fig. 9. Survival rates of recipients (LEW rats) of SAL-incubated allogeneic (CAP) bone marrow (——), N = 43. Comparison with recipients of untreated BM (. . .), N = 41, and control animals which were merely irradiated (– – –), N = 50

2. Lymphatic Tissue Restitution

In these animals the histologic appearance of the various lymphatic tissues was examined after 2–4, 6–8, 12, 26 or 52 weeks. Furthermore, they were tested for chimerism with cytotoxic hyperimmune alloantisera LEW-anti-CAP and the reverse, using the two-step cytotoxicity test with fresh rat serum as the source of complement.

The thymus of recipients of SAL-incubated BM revealed complete restitution after 6 weeks (Fig. 10b) and maintained this appearance. In contrast, the few surviving recipients of untreated BM showed complete atrophy of this organ (Fig. 10a). As shown in the figure, beneath a broad fibrous capsule only some epithelial cells and lipidladen macrophages became visible.

The lymph nodes of recipients of SAL-incubated BM also showed normal appearance after 6 weeks. As can be seen in Fig. 11b, the typical large follicles and germinal centers beneath the marginal sinus and lymphocyte-rich thymus dependent areas were visible. Thus, as in the thymus, no signs of GVHR were

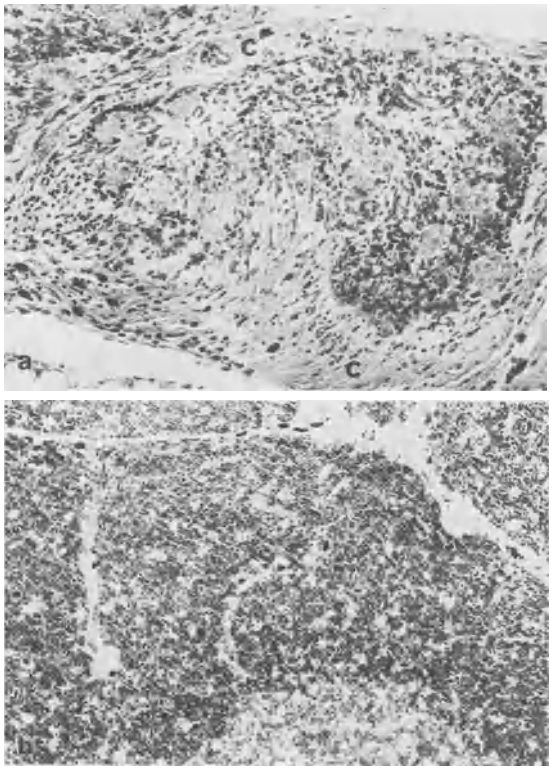


Fig. 10. Thymus morphology in rats 6 weeks after lethal irradiation and transplantation of allogeneic BM: **a** in a recipient of untreated BM; **b** recipient of SAL-incubated BM. Giemsa; 160×; c=fibrous capsule

seen, similarly not at later times. In the other hand, as to be expected, the lymph nodes of recipients of untreated BM showed typical signs of GVHR (Fig. 11a): enlargement and lymphocyte depletion of the paracortical thymus-dependent area with infiltration by immunoblasts and light-stained macrophages, and lymphocyte depletion of the follicular area.

The chimerism test data of reconstituted animals are summarized in Fig. 12. The percentage of donor type cells was still low after 4 weeks, it then increased until week 7–8 and remained remarkably constant thereafter at a 50–60% level.

3. Immune Reactivity

Thus far, reconstituted recipients of SAL-incubated BM were mainly tested for cellmediated reactivity in vivo, by skin grafting. As can be seen in Fig. 13, grafts from MHC-different and even those from non-MHC-different third party strains were rejected in a normal fashion. This indicates restitution of immune reactivity to normal 6–7 weeks after BM grafting.

Preliminary tests (N=5) for humoral antibody reactivity after immunization with sheep red blood cells showed development of similar antibody titers as untreated animals of the same inbred strain.

Specific unresponsiveness against BM donor strain transplantation antigens, as to be expected from the above chimerism data, was proven by unlimited survival of skin grafts in all cases tested (observation time > 1 year).

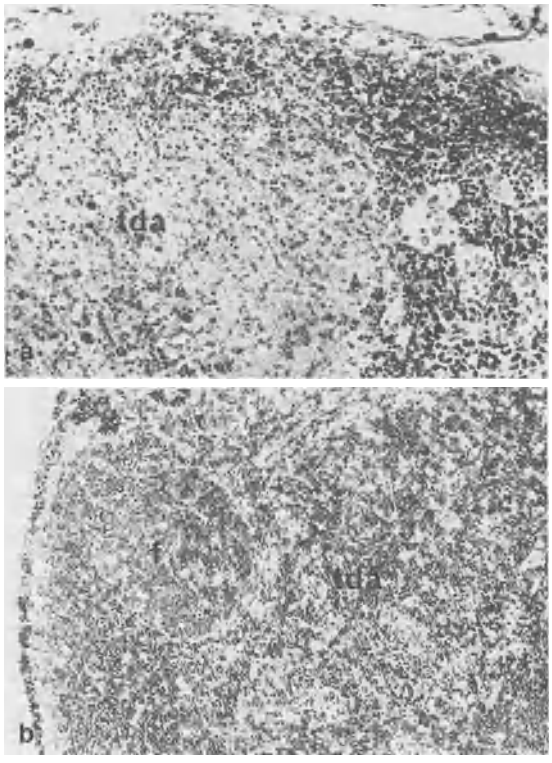


Fig. 11. Lymph node morphology in rats 6 weeks after lethal irradiation and transplantation of allogeneic BM: **a** in a recipient of untreated BM; **b** recipient of SAL-incubated BM. Giemsa; 160×; f=follicular area; tda=thymus dependent area

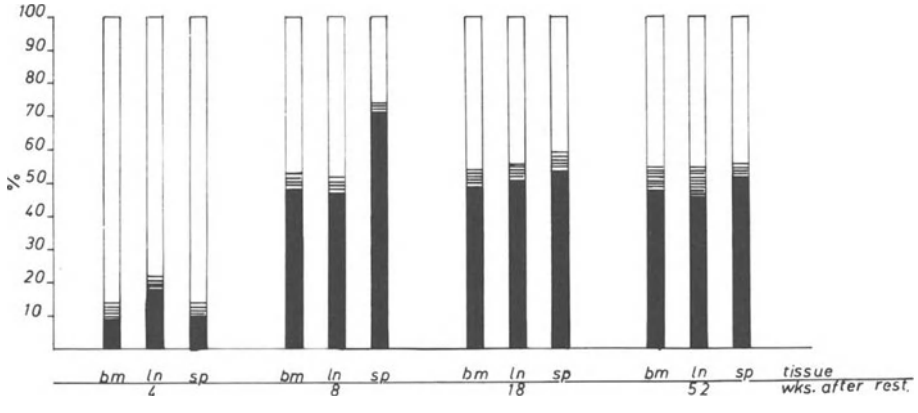


Fig. 12. Chimerism in recipients of SAL-incubated allogeneic bone marrow. Percentage of donor cells in bone marrow (BM), lymph nodes (LN) and spleen (SP) 4, 8, 18 and 52 weeks after restitution. Solid part of each column: lower limit value; hatched part: range of measured values. N=14. The cells were incubated with cytotoxic hyperimmune alloantisera of recipient$\rightleftarrows$donor strain combinations. Test details: see Figure 3

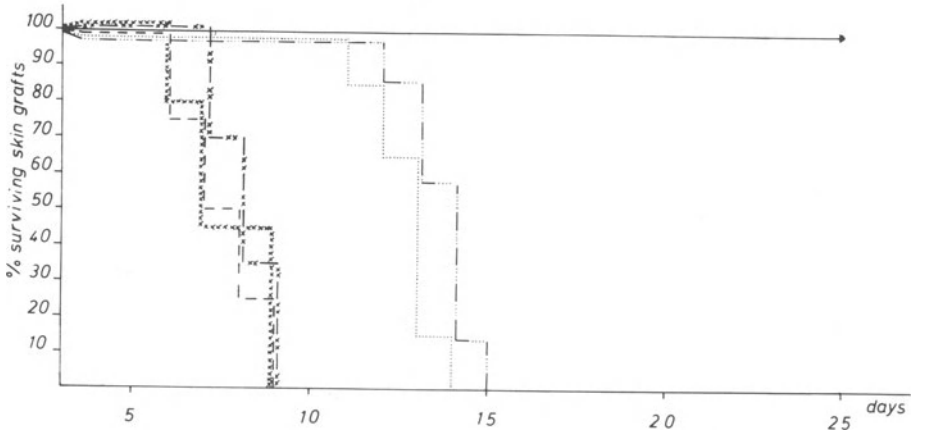


Fig. 13. Immune reactivity of recipients of SAL-incubated allogeneic bone marrow. Survival times of skin allografted from third party strains and from the BM donor strain 6 weeks after restitution: MHC histoincompatible BDV→ restituted LEW (—— + + ——), N=10. MHC histoincompatible BDV→ normal control LEW (+ + +). Non-MHC histoincompatible F 344 → restituted LEW (—— . . . ——), N=15. Non-MHC histoincompatible F 344 → normal control LEW (. . .). BM donor CAP → restituted LEW (——), N=15. BM donor CAP → normal control LEW (– – –)

II. BM Pretreatment with SAT

To determine whether similar results to those described in section I could also be obtained with BM grafts which lack postthymic cells but still contain prethymic precursors, SAL was replaced by SAT.

1. Recipient Mortality

The survival rates in this series are presented in Fig. 14. The great majority of recipients of SAT-incubated BM died. But in comparison with recipients of untreated BM, deaths started with a delay of 3 weeks. Their general appearance indicated the occurrence of GVHR.

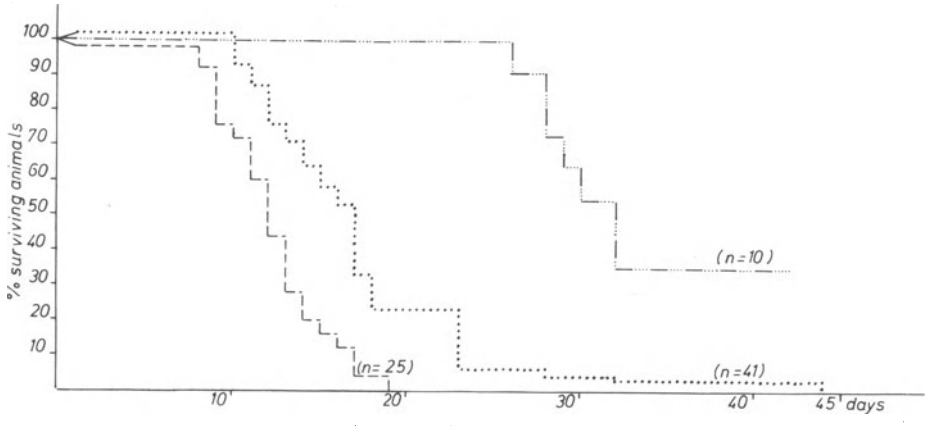


Fig. 14. Survival rates of recipients (LEW rats) of SAT-incubated allogeneic (CAP) bone marrow (—— . . . —— . . .), N=10. Comparison with recipients of untreated BM (. . .), N=41, and control animals which were merely irradiated (– –), N=50

2. Histological Appearance of Lymphatic Tissue

Five to 6 weeks after BM grafting, thymus, lymph nodes and spleen were thoroughly evaluated histologically for signs of GVHR.

The thymus morphology is shown in Fig. 15. The recipients of SAT-incubated BM (i.e. of an allogeneic BM which still contains prethymic precursors), reveal a thymus cortex which is depleted of lymphocytes (Fig. 15a). Between cortical epithelial cells some lipid-laden macrophages became visible; the intrathymic perivascular spaces are enlarged and filled with macrophages. This appearance strongly contrasts with the normal thymic structure of a recipient of SAL-incubated BM (Fig. 15b).

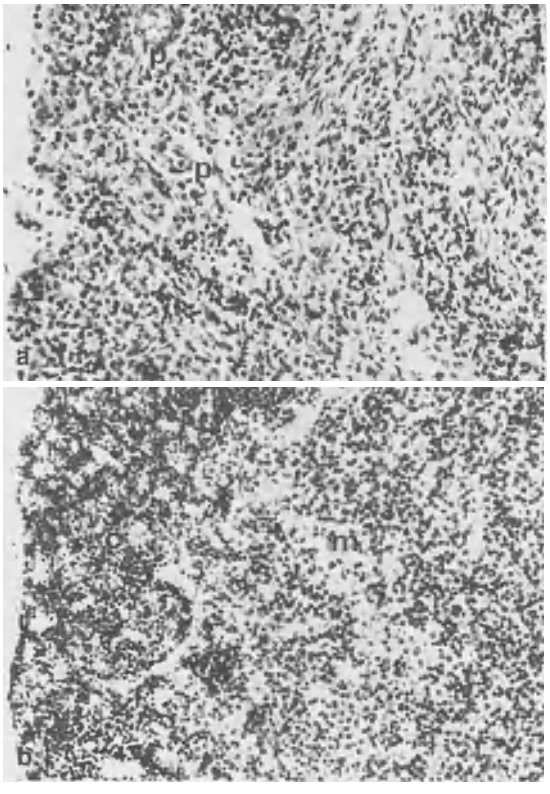


Fig. 15. Thymus morphology in rats 5–6 weeks after lethal irradiation and transplantation of allogeneic BM: **a** in a recipient of SAT-incubated BM; **b** recipient of SAL-incubated BM. H & E; 128×; c=cortex; m=medulla; p=perivascular space

The lymph node morphology is presented in Fig. 16 and corresponds with that of the thymus. Again, in recipients of SAT-incubated BM typical signs of GVHR were found: The lymphocyte depletion of the thymus-dependent area and the other symptoms (Fig. 16a) are quite similar to those observed after grafting of untreated BM (see also Fig. 11a). The contrast to the normal lymphocyte content of the thymus dependent area and the presence of submarginal lymph follicles in recipients of SAL-incubated BM (Fig. 16b) is obvious.

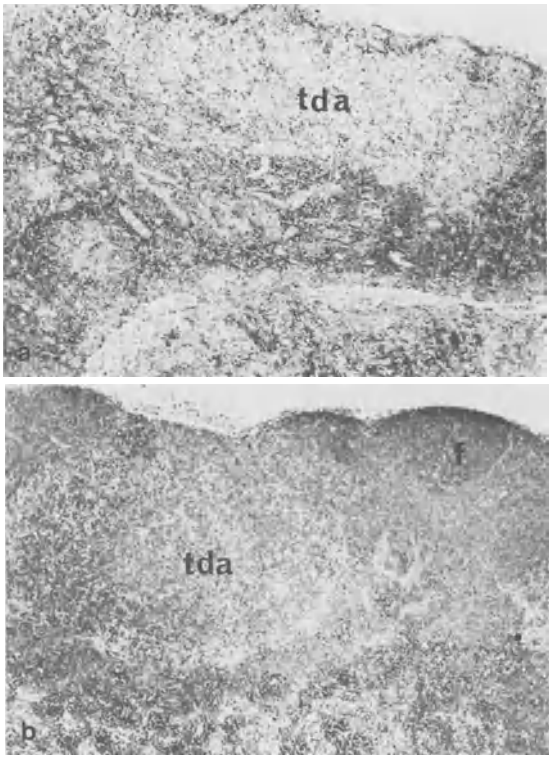


Fig. 16. Lymph node morphology in rats 5–6 weeks after lethal irradiation and transplantation of allogeneic BM: **a** in a recipient of SAT-incubated BM; **b** recipient of SAL-incubated BM. Giemsa; 64×; f = follicular area; tda = thymus dependent area

3. Characterization of Hemolytic Anemia

A characteristic feature of the GVHR with delayed onset, which was observed in the recipients of SAT-incubated BM, consisted of the following morphological and functional signs of hemolytic anemia: In spleen sections erythrophagocytosis and rich iron deposits in macrophages were found (Fig. 17a). Hematocrits in animals dying in the fifth week postgrafting were 18–22%, in animals killed after 6 weeks 30–36% (normal values: ~45%). Direct antiglobulin tests were positive 1:16 – 1:32 with anti-IgG and 1:2 – 1:4 with anti-IgM.

F. Restitution of Xenografted BM Recipients

To determine whether the BM pretreatment which successfully allowed GVHR-free allografting, namely SAL-incubation, would also enable xenotransplantation of hemopoietic stem cells, following experiments were conducted: 5×10^7 SAL-incubated CAP rat BM cells were injected i.v. into 12–14 week old female mice 1 and 18 hrs after lethal irradiation with 950 rads from a ^{60}Co source. In most experiments BALB/c mice were used as BM recipients, but similar findings were also obtained with C3H mice.

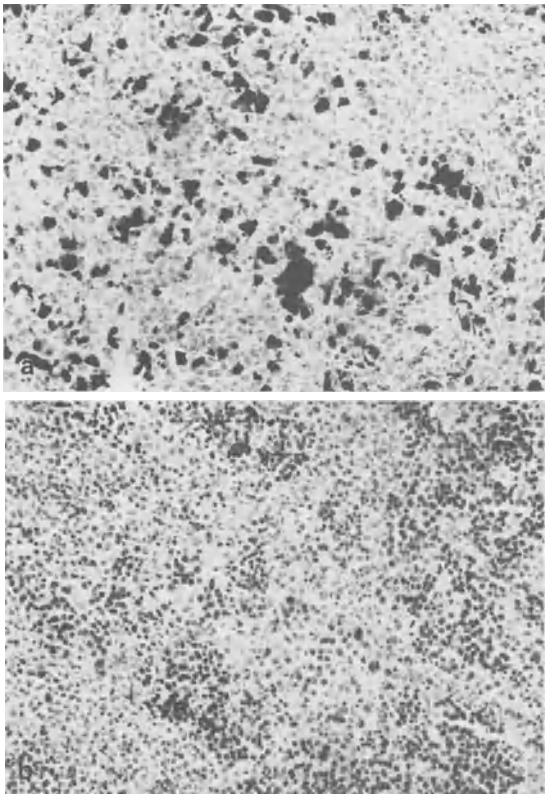


Fig. 17. Spleen morphology in rats 5–6 weeks after lethal irradiation and transplantation of allogeneic BM: **a** in a recipient of SAT-incubated BM; **b** recipient of SAL-incubated BM. Prussian blue; 128×; black spots = iron deposits

I. Recipient Survival

The survival rates are presented in Fig. 18. Similar to the rat experiments (see Fig. 9), irradiation controls survived only 10–25 days and recipients of untreated BM also died within the same range of time. However, the latter did not die from the lack of hemopoiesis but with typical signs of an acute GVHR, most clearly indicated by the histological evaluation of those animals which survived longer than 20 days (see below). In contrast, most recipients of SAL-incubated BM survived the whole period of observation in good health, now extending to > 35 weeks. Again similar to the rat experiments, only a small percentage died during the third week of the irradiation recovery phase, from infections due to the conventional housing conditions.

II. Lymphatic Tissue Restitution

Mice of the control group which had received untreated rat BM exhibited typical signs of GVHR. The thymus showed a severe atrophy with enlargement of perivascular spaces (Fig. 19b) and in lymph nodes an almost complete atrophy of the cortical tissue was also seen, although plasma cells in the medullary cords were

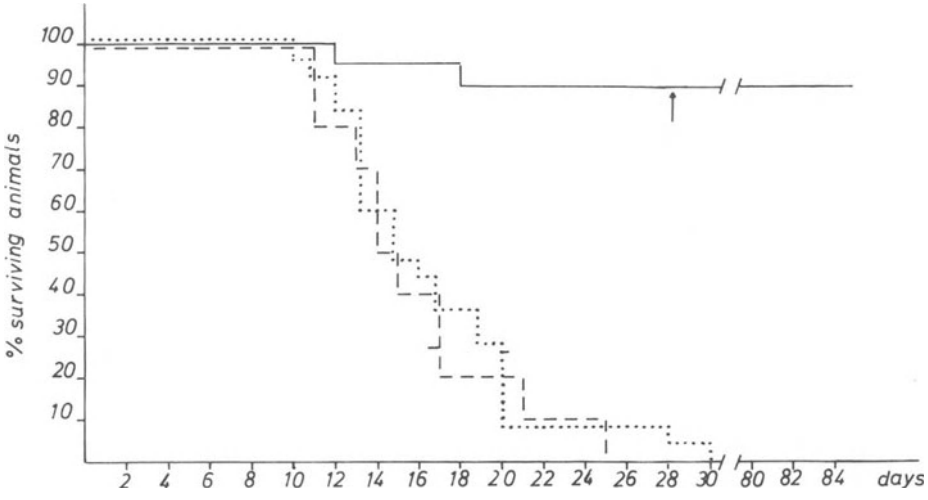


Fig. 18. Survival rates of recipients (BALB/c mice) of SAL-incubated xenogeneic (CAP rat) bone marrow (——), N=42 until day 30, N=20 for non-killed long-term survivors. Comparison with recipients of untreated BM (. . .), N=47, and control animals which were merely irradiated (– – –), N=38

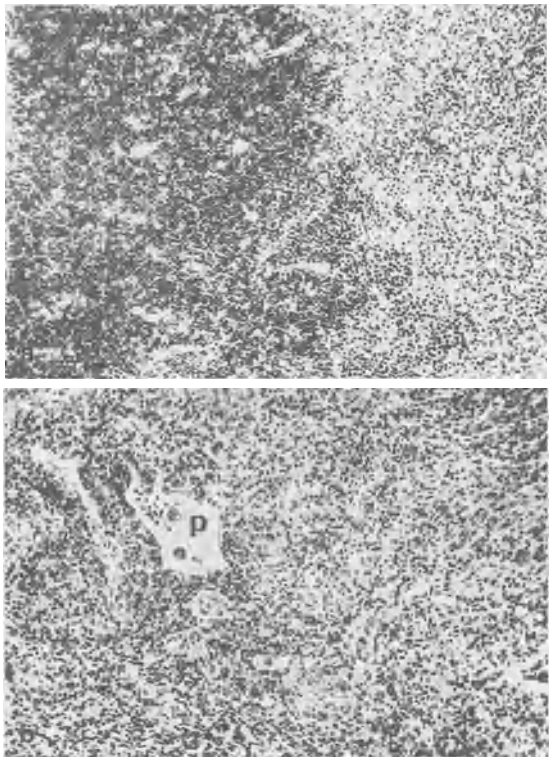


Fig. 19. Thymus morphology in mice 5 weeks after lethal irradiation and transplantation of xenogeneic (rat) BM: **a** in a recipient of SAL-incubated BM; **b** recipient of untreated BM. Giemsa; 128×; p=perivascular space

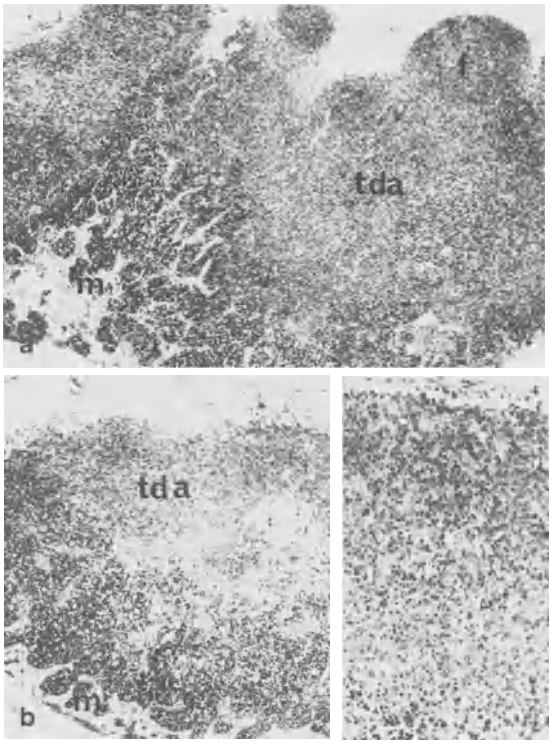


Fig. 20. Lymph node morphology in mice 5 weeks after lethal irradiation and transplantation of xenogeneic (rat) BM: **a** in a recipient of SAL-incubated BM; **b** recipient of untreated BM. Inset in **b**: Higher magnific. of cortical tissue. Giemsa; 64× and 160×; f=follicular area; tda=thymus dependent area

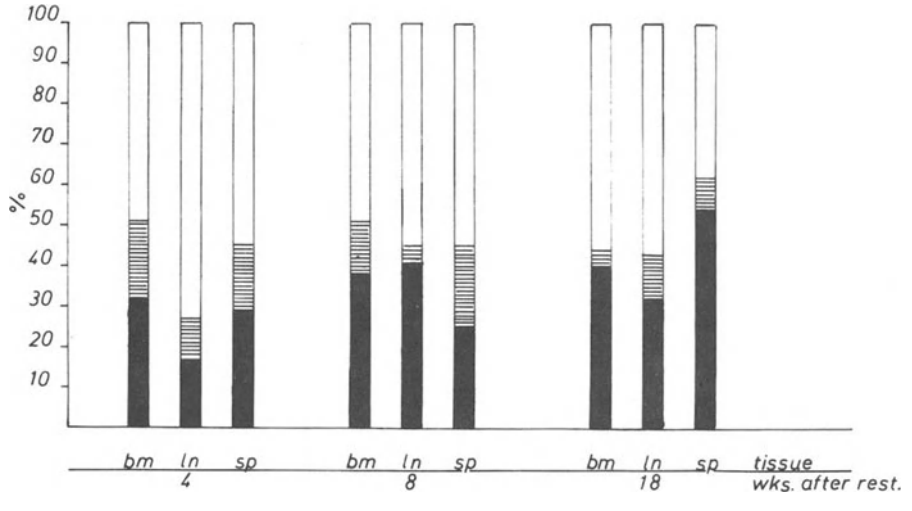


Fig. 21. Chimerism in mice recipients of SAL-incubated xenogeneic bone marrow. Percentage of donor cells in bone marrow (BM), lymph nodes (LN) and spleen (SP) 4, 8 and 18 weeks after restitution. Solid part of each column: lower limit value; hatched part: range of measured values; n=13. The cells were incubated with cytotoxic xenoantisera of the recipient → donor combination

about as numerous as normally (Fig. 20b). At higher magnification the cortical tissue showed numerous basophilic blast cells in the submarginal region.

After about 5 weeks the reconstituted recipients of SAL-incubated xeno BM exhibited an almost normal restitution of thymus morphology (Fig. 19a). Correspondingly, their lymph nodes revealed about normal organization and lymphocyte content, as seen in Fig. 20a.

These reconstituted lymphatic tissues were tested for chimerism with strongly cytotoxic mouse (recipient strain)-anti-CAP rat-sera. As shown in Fig. 21, a significant percentage of the cells was found to be of donor origin. At 4 weeks these figures were already higher than in the analogous rat chimera. As a whole, they levelled in the order of 40–50% over an extended period of observation.

III. Immune Reactivity

As with the allo-chimeras, reconstituted xeno-chimeras were tested after 6 weeks by measuring skin graft survival times. CAP rat ear skin and H-2-incompatible mouse control allografts were applied to the ventral thoraco-abdominal region. As shown in Fig. 22, the third party allografts were rejected normally when compared to allograft survival times on normal mice in the same strain combination. This indicates recovery of immunological reactivity.

At the same time specific unresponsiveness against BM donor transplantation antigens was indicated by permanent survival of BM donor skin allografts (observation time > 200 days).

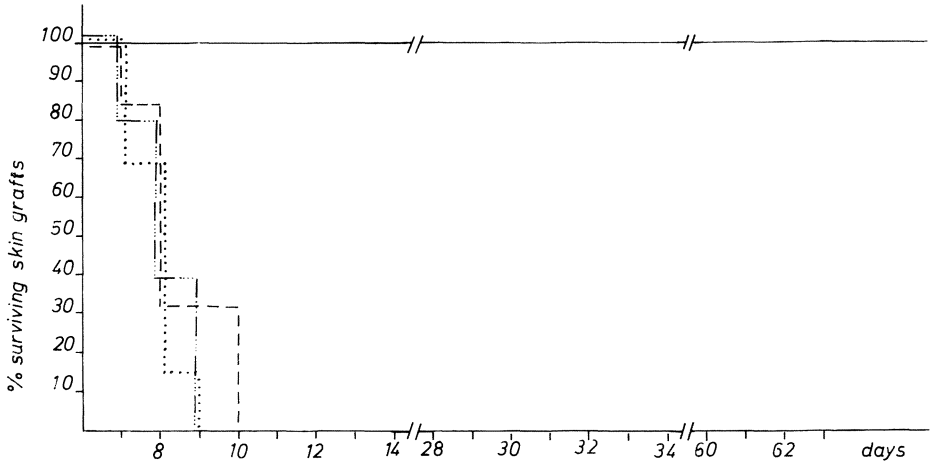


Fig. 22. Immune reactivity of recipients of SAL-incubated xenogeneic bone marrow. Survival times of skin grafted from a third party mouse strain and from the BM donor rat strain 6 weeks after restitution: MHC histoincompatible C3H → restituted BALB/c (– – –), n=12. MHC histoincompatible C3H → normal control BALB/c (–...–). BM donor CAP → restituted BALB/c (——), n=20 BM donor CAP → normal control BALB/c (...)

G. Discussion and Conclusions

The results described demonstrate the usefulness and remarkable efficiency of the principle of serological dissection upon which our approach to BMT is based. It was introduced in our laboratory in 1970 (Kluck et al., 1971). Provided, that the appropriate antisera are available, this dissection of lymphocytes by in vitro incubation at 37° C is a fast procedure which was extended from 30 min. in our earlier experiments to 45 min. only for reasons of safety. Though it has not yet been tested, one may expect that the addition of complement is not required.

Much effort had to be spent to prepare a serum exhibiting the appropriate selective activity from a crude, polyspecific antilymphocyte serum. It was considered essential to prove that real specifity increases take place by the serologically exhaustive absorptions with cross-reactive cells, rather than just a reduction of antibody titer, which is another consequence of any absorption procedure. To this end the multiple in vitro and in vivo experiments presented in chapters C and D were performed. However, the aim was not to prepare a purely antilymphocytic antiserum: only those antibodies which crossreact with hemopoietic cells had to be removed completely (any further purification attempt might even have reduced the antiserum effectiveness unnecessesarily). An alternative possibility would be the preparation of appropriate monoclonal antibodies.

The network of cell surface structural relationships between related cells, as revealed by the absorption analysis, suggests that lymphocytes are antigenically more closely related to hemopoietic cells when the latter are less mature. This is in line with the contention that 1. lymphocytes are descendents of hemopoietic stem cells and 2. each line of hemopoietic differentiation includes a loss or hiding of some and the appearance of other cell surface structures.

SAT was prepared from the same antilymphocyte serum as SAL, thus allowing a most informative comparison. It may be provisionally defined by its pattern of reactivity against various cell types:

non-lymphoid hemopoietic cells	–
not-thymus-processed lymphocytes	–
thymocytes	$\sim$100%+
lymph node lymphocytes	$\sim$60–70%+.

This pattern is remarkably similar to that which has recently been described by Wonigeit (1969) and termed RT-Ly-1. It is dissimilar to Thy-1 which shows a quite different cell distribution in rats (Arndt et al., 1977; Hunt et al., 1977) and would have been removed at the latest by absorption with fetal liver cells.

GVHR following BMT has been studied mainly in mice, using parent/F_1 hybrid combinations. Even in H-2-different strain combinations the onset is usually delayed and mostly far below 100% (Uphoff, 1976). Thierfelder and Rodt (1977) have called these deceptively easy systems as far as the suppression of GVHR is concerned, pointing out that it is not entirely clear why is this so. In contrast, the rat model used in our studies presented itself as severe in terms of GVHR: Nearly all recipients of untreated allogeneic BM died from acute reactions. This does not appear to be a special feature of the CAP $\rightarrow$ LEW strain

combination, since the same was found in 4 other RT1-histodifferent combinations (unpublished data). Therefore it may be argued that these rat models, different from those with mice, provide good models for the type of BM-induced GVHR which is also observed in the human.

The symptomatology of GVHR is well-known though often not fully enough considered. The so-called mortality assay (Korngold and Sprent, 1978) naturally suffices to answer the question how many animals die from GVHR; it certainly does not suffice when one is asking for prevention of GVHR. A variety of functional symptoms is useful for the evaluation of sublethal reactions of various degrees of severity. However, based on our experience over several years we strongly advise the inclusion of histological studies of various tissues and organs, particularly thymus, lymph nodes and spleen, but also Peyer patches, liver, intestinal wall, skin etc. Such morphological evaluations appear to provide the most sensitive measures of GVHR and, therefore, would allow far reaching conclusions in terms of prevention of GVHR.

This is one of the major aspects to be kept in mind when considering the literature on BMT following preincubation of the BM in anti-T cell sera, be they allogeneic (such as anti-Thy-1 in mice) or xenogeneic antisera (see also Müller-Ruchholtz et al., 1978). Another important factor, the kind of animal combination used, was mentioned above. Taken together the data indicate that it is a question of the model and the methodology how far the development of GVHR, after incubation of BM in anti-T cell-sera, is detected. This view is further supported by the findings described in chapter E II. These were obtained in a fully allogeneic MHC-different system, i.e. a system in which no one has been successful so far with an SAT.

The findings that SAT does not prevent GVHR do not support the contention of Zinkernagel (1978) that it is the thymus which determines T cell specificity for self. They rather indicate that at least reactivity against MHC transplantation antigens is already irreversibly determined in prethymic precursors. They would not be incompatible, however, with a less stringent view which may be derived from recent reports of Fink and Bevan (1978) or Katz et al. (1978) about determination of preferential (i.e. better) immune responsiveness of T-cells against various antigens by the MHC-type of resident cells in the host thymus: MHC restriction simply optimises the reactivity against certain antigens. On the other hand, the in vivo relevance of such restriction of allograft rejection has been severely questioned (Murasko, 1978).

The delay of GVHR following SAT-incubation of BM may be explained by the time prethymic precursors require for the development of functional capacity to give rise to immune reactions. The delay period of about 3 weeks observed in our experiments is the same as reported by several authors (see Cantor and Weissman, 1976) for the maturation of prethymic precursors in mouse BM to mount specific MLR, GVHR or cell-mediated immune cytolysis in vitro. This delay may also explain the appearance of hemolytic anemia which was not observed in the cases of acute GVHR following grafting of untreated BM: There was sufficient time for recipient's B cells to recover from irradiation and to initiate the autoimmune hemolytic anemia triggered by the donor's T cells, as so clearly analysed by the Gleichmanns (1976) in relevant mouse models.

The SAL activity differed from SAT activity in that SAL was reactive against the various lymphocyte subpopulations and differentiation stages. What we were mainly interested in was evidence of its reactivity against prethymic precursors and therefore the data presented in chapter DI were collected. From the titer drop SAL → SAT by a factor of 8 (see chapter CI1) and the information that the antibodies removed were mainly reactive with Ig cells (Arndt et al., 1977), it may be concluded that the great majority of SAL antibodies are directed against antigenic determinants which are present on immature lymphocytes, such as the prethymic precursors.

In earlier experiments GVHR-prevention titers of SAL were found to be much higher than the titers measured in serological in vitro cytotoxicity tests. A thymocytotoxic titer of 1:64 (defined by death of 50–60% of the cells) corresponded with a prevention titer of 1:512, since preincubation of CAP spleen cells in antiserum of this dilution still prevented GVHR upon their injection into neonetal LEW rats in 8/11 experiments. Therefore, our standard procedure of incubating BM in undiluted SAL provides a wide margin of safety.

SAL appears to provide a tool to distinguish the undetermined stem cell potential from that of lymphocytically determined cells, also in the adult to a degree which, thus far, could not even be reached in experiments in the newborn. (Remember that B type lymphocytes can already be demonstrated in the 12-day-old mouse embryo, see Melchers, 1977.) This new approach represents a principle of indirect manipulation of stem cells, and thereby, explains the favourable results obtained and described above. They have recently been confirmed at the Sloan-Kettering Institute (R. A. Good, personal communication).

The chimerism observed in these experiments, at a remarkably constant level of around 50%, rather than a 95–100% donor type BM and lymphoid chimerism, is in contrast to the observations in several other studies with radiation chimeras. The most plausible explanation for these findings is that in our experiments no GVHR has occurred whereas 100% donor type chimerism may be considered as a consequence of a subclinical GVHR that essentially wipes out recovering host cells, since it is unlikely that lethal whole-body irradiation kills all hemopoietic stem cells of the recipient.

The restitution of immune reactivity described here is in contrast to that observed by others in long-term survivors of GVHR of varying degrees of severity following BM allografting, e.g. in mouse (Gengozian et al., 1971) and man (Noel et al., 1978), where longlasting immunodeficiencies were found. Turned around, this difference may serve as an additional argument for absence of GVHR in our experiments. This argument may be the more valid as the detectability of immunodeficiencies is dependent on the test antigen used in that minor grades may remain undetected when testing with strong antigens only: For this reason not only MHC-different but also non-MHC-different third party skin allografts were tested for their survival times.

Xenotransplantation poses non-immunological and immunological problems additional to those of allotransplantation (Milgrom and Najarian, 1975): 1. In crossing the species lines strong histocompatibility barriers may be encountered, additional to MHC-coded allo-barriers which have also been shown to be

operational in xenogeneic reactivity (Boylston et al., 1978). 2. Preexisting natural antibodies, and possibly also cell-mediated immunity (Yoshioka et al., 1977), to foreign species antigens are to be expected, the stronger the more phylogenetically distant the species are. Therefore, it may be interesting that, to our knowledge, the observations described in chapter F provide the first successful xenogeneic BM transplantation, using the principle of a simple in vitro serological dissection of BM. This appears to be at least as effective as a procedure recently published by Pierpaoli and Maestroni (1978) which required extensive additional pre- and post-conditioning treatments with a special set of 3 immunosuppressive drugs to prevent GVHR.

Taken together, the experiments reported here indicate a flexibility of hemopoietic stem cells and their lymphoid differentiating offspring in adaptation to a foreign environment which we would not have dared to predict. However, we are well aware of the many questions which are still open and which are to be posed in consequence, though it is not intended to enumerate them here.

In summary, the following six concluding points may be given:

1. Natural xenogeneic antilymphocyte sera react with a variety of BM cells and allow the detection of various patterns of antigenic determinants on the surface of different hemopoietic cells.
2. Adequate combinations of absorptions remove all antibodies cross-reactive with hemopoietic cells. Thus, an incubation of BM in specified antisera leaves stem cells unaffected.
3. These antisera may be specified antilymphocyte (SAL), i.e. cytotoxic for BM lymphocytes including prethymic precursors, or further specified anti-T (SAT). Thus, a short (45 min.) incubation of BM selectively kills the respective lymphoid cells.
4. Fully allogeneic, MHC-different adult rat BM allows normal restitution, establishment of permanent chimerism, recovery of immunological reactivity and specific nonreactivity when pretreated with SAL, whereas recipients of untreated BM die from acute GVHR.
5. The same BM induces delayed and mostly fatal GVHR when pretreated with SAT, i.e. prethymic cells differentiate irreversibly and react against their MHC-different environment.
6. Xenogeneic, SAL-incubated BM (rat → mouse) also allows normal restitution, similar to the allogeneic rat model, again indicating a remarkable flexibility of hemopoietic stem cells and their differentiating offspring in adaptation to their environment.

References

Arndt, R., Thiele, H.-G., Stark, R., Wottge, H.-U., Müller-Ruchholtz, W.: Analysis of the antigenic structure of the thymocyte surface membrane by heterologous anti-thymocyte serum. Eur. J. Immunol. *7*, 131–136 (1977)

Boylston, A. W., Anderson, R. L.: Human-mouse mixed lymphocyte cultures. Immunology *35*, 455–461 (1978)

Cantor, H., Weissman, I.: Development and function of subpopulations of thymocytes and T lymphocytes. Progr. Allergy *20,* 1–64 (1976)

Elkins, W. L.: Cellular immunology and the pathogenesis of graft versus host reactions. Progr. Allergy *15,* 78–187 (1971)

Fink, P. J., Bevan, M. J.: H-2 antigens of the thymus determine lymphocyte specificity. J. Exp. Med. *148,* 766–775 (1978)

Gengozian, N., Congdon, C. C., Allen, E. A., Toya, R. E.: Immune status of allogeneic radiation chimeras. Transplant. Proc. *3,* 434–436 (1971)

Gleichmann, E., Gleichmann, H.: Graft-versus-host-reaction: a pathogenetic principle for the development of drug allergy, autoimmunity, and malignant lymphoma in non-chimeric individuals. Z. Krebsforsch. *85,* 91–109 (1976)

Goldschneider, I., Gordon, L. K., Morris, R. J.: Demonstration of Thy-1 antigen on pluripotent hemopoietic stem cells in the rat. J. Exp. Med. *148,* 1351–1366 (1978)

Hunt, S. V., Mason, D. W., Williams, A. F.: In rat bone marrow Thy-1 antigen is present on cells with membrane immunoglobulin and on precursors of peripheral B lymphocytes. Eur. J. Immunol. *7,* 817–823 (1977)

Katz, D. H., Skidmore, B. J., Katz, L. R., Bogowitz, C. A.: Adaptive differentiation of murine lymphocytes. I. J. Exp. Med. *148,* 727–745 (1978)

Kluck, A., Sonntag, H.-G., Müller-Ruchholtz, W.: Selektive Inaktivierung immunokompetenter Zellen aus Zellsuspensionen durch in-vitro-Behandlung mit lymphozytotoxischen Antikörpern. In: 3. Tgg. Ges. Immunol., p. 2. Marburg: Eukerdruck 1971

Komuro, K., Boyse, E. A.: Induction of T cells from precursor cells in vitro by a product of the thymus. J. Exp. Med. *138,* 479–482 (1973)

Korngold, R., Sprent, J.: Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. J. Exp. Med. *148,* 1687–1698 (1978)

Melchers, F.: B lymphocyte development in fetal liver. I. Development of reactivities to B cell mitogens "in vivo" and "in vivo". Eur. J. Immunol. *7,* 476–481 (1977)

Milgrom, F., Najarian, J. S.: Cross-reacting antigens and heterotransplantation. Transplant. Proc. *7,* 885–886 (1975)

Müller-Ruchholtz, W., Wottge, H.-U., Müller-Hermelink, H. K.: Selective grafting of hemopoietic cells. Transplant. Proc. *7,* 859–862 (1975)

Müller-Ruchholtz, W., Wottge, H.-U., Müller-Hermelink, H. K.: Bone marrow transplantation in rats across strong histocompatibility barriers by selective elimination of lymphoid cells in donor marrow. Transplant. Proc. *8,* 537–541 (1976)

Müller-Ruchholtz, W., Wottge, H.-U., Müller-Hermelink, H. K.: Modulation of immune reactions by antilymphocyte serum. Transplant. Proc. *10,* 23–29 (1978)

Murasko, D. M.: Apparent lack of H-2 restriction of allograft rejection. J. Immunol. *121,* 958–961 (1978)

Noel, D. R., Witherspoon, R. P., Storb, R., Atkinson, K., Doney, K., Mickelson, E. M., Ochs, H. D., Warren, R. P., Weiden, P. L., Thomas, E. D.: Does graft-versus-host disease influence the tempo of immunologic recovery after allogeneic human marrow transplantation? An observation on 56 long-term survivors. Blood *51,* 1087–1105 (1978)

Pierpaoli, W., Maestroni, G. J. M.: Drug-induced chimerism and prevention of graft-versus-host disease in lethally irradiated mice transplanted with rat bone marrow. Transplantation *26,* 456–458 (1978)

Simonsen, M.: Graft versus host reactions. Their natural history, and applicability as tools of research. Progr. Allergy *6,* 349–467 (1962)

Thierfelder, S., Rodt, H.: Antilymphocytic antibodies and marrow transplantation. Transplantation *23,* 87–92 (1977)

Uphoff, D. E.: Mechanisms in graft-versus-host reactions: An immunogenetic appraisal. Transplant. Proc. *8,* 515–519 (1976)

Wonigeit, K.: Definition of lymphocyte antigens in rats: RT-Ly-1, RT-Ly-2 and a new MHC linked antigen system. Transplant. Proc. *11,* 1334–1336 (1979)

Yoshioka, H., McCalman, R. T., Putnam, C. W., McIntosh, R. M. Terman, D. S.: Attenuation of hyperacute xenograft rejection in unmodified host by extracorporeal plasma perfusion. Transplantation *24,* 78–81 (1977)

Zinkernagel, R. M.: The thymus: its influence on recognition of "self major histocompatibility antigens" by T cells and consequences for reconstitution of immunodeficiency. Springer Semin. Immunopath. *1*, 405–415 (1978)

Discussion

Kersey: Have you ever tried to perform these experiments with bone marrow incubated with antisera but without adding complement? Second question concerning the difference between SAL and SAT: You concluded that you did not kill prethymic cells with the SAT wheras apparently thought you did with SAL. I wonder if you have any data to support that or if alternatively it is possible that in fact there may be cells other than T cells involved in the gvh reaction.
Müller-Ruchholtz: We have not used bone marrow incubated in antisera without complement. To your second question: I cannot rule out the possibility that other than T cells participate in gvh, but the patterns of early(!) histological restitution appearance which we see using SAL or SAT was alike.
Thierfelder: Could you elaborate a little more on the specificity of your SAL. Is it an antiserum which reacts with thymocytes, is it an antiserum which reacts with T and/or B cells. What is the specificity as to the T cell population.
Müller-Ruchholtz: The specificity of the SAL includes both thymocytes T cells and B cells.
Thierfelder: I shall present some data where we found that classical anti-thymocyte serum interferes with engraftment of rat bone marrow. Therefore if SAL reacts with a thymocyte antigen from our experience we would have expected that there was no take.
Müller-Ruchholtz: I should add that this SAL cannot include anti-Th-1 reactivity because of our preabsorptions
Hunt: Which absorption would remove anti-Th-1 activity?
Müller-Ruchholtz: Certainly not the absorption with erythrocytes, questionably the absorption with granulocytes and macrophages, very probably the absorption with fetal liver cells.
van Bekkum: If your SAL presumably knocks out the prethymic cell how then can reconstitution of your chimeras occur from the stem cells without inducing GVH. It does not make any difference to me whether your donor prethymic cell is transplanted and then has to enter the thymus etc., or whether it is generated by the pluripotent stem cell.
Müller-Ruchholtz: My philosophy is this: SAT eliminates the differentiated T cells, the prethymic T cells are still present and capable to mount GVH. SAL eliminates all lymphocytes and the lymphocytes developing from the undetermined stem cells develop tolerance towards the host.

Influence of the Recipient Thymus on the Maturation of T-Lymphocytes in H-2 Different Radiation Chimeras

H. Rodt, S. Thierfelder, and G. Hoffmann-Fezer

A. Summary

Graft-versus-host reactions in mice can be suppressed by incubation of the donor cells with T-cell-specific antibodies prior to transplanting them into lethally irradiated recipients. Since reconstitution of the immune functions in these recipients requires the formation of a new T-lymphocyte population, we investigated whether H-2 differences between recipient thymus and donor stem cells as well as an age dependent thymic involution impaired the maturation of immunocompetent T-lymphocytes. For this purpose the immune response of different chimeric mice against SRBC and third party skin grafts as well as the repopulation of the lymphatic organs with T-lymphocytes was evaluated. Chimeras of the type CBL → CBL/CBA and CBL/CBA → CBA were used for the experiments. In addition chimeras which had been thymectomized, transplanted with the thymus of one parent strain, lethally irradiated and transfused with bone marrow from the other parent strain were analyzed. In all chimeric combinations, even in the case of complete H-2 difference between donor cells and recipient thymus, a normal or almost normal reconstitution of the immune response against SRBC and incompatible skin grafts was detected. Furthermore age-involuted thymi returned to normal morphologically and functionally after bone marrow transplantation. The repopulation of the lymphatic organs in the investigated chimeric mice was remarkably similar to normal controls. This indicates that hemopoietic stem cells and/or lymphopoietic progenitors may differentiate in a thymus in spite of H-2 differences to functional T-lymphocytes able to respond against SRBC and incompatible skin grafts.

B. Introduction

Graft-versus-host disease (GVHD) induced by injection of histoincompatible marrow cells into lethally irradiated recipients can be abrogated or greatly modified by pretreatment of the donor cell inoculum with xenogeneic antisera specifically directed against T-lymphocytes. Mice given H-2 incompatible, antiserum-incubated spleen cells survived without GVHD, whereas recipients of untreated cells died after a short period [13, 12, 17]. Recently it has been shown, that an incubation with T-cell specific antibodies has also been successful to prevent GVHD in dogs [7, 11] and probable in clinical marrow transplantation [11]. Since this approach is based on the transfer of T-cell deprived marrow cells, the restauration of the immunological defense mechanisms require the reconsti-

tution of the T-cell dependent reactivity by the recipient. However, several authors have claimed that the immunological capability of long term radiation chimeras may be impaired with respect to the qualitative and quantitative character of the responses to the challenge with certain antigens [9, 8, 19]. Several reasons for the impairment of the immunological functions have been discussed. It is known, that chronic GVHD is associated with protracted immunosuppression [9]. Normal immune reactivity may further decline with the age of the tested animals caused by involution of the thymic tissues [3]. An even more critical question is whether lymphopoietic progenitor cells may differentiate in a H-2 different thymus to mature T-lymphocytes acquiring a recognition structure with specificity for histocompatibility and foreign antigens [21]. Data presented in this paper are an approach to analyze the reconstitution of the T-cell-dependent reactivity and the repopulation of the lymphatic organs with T-lymphocytes in several H-2 incompatible chimeric combinations. Two different aspects of the immune response of the chimeras have been studied: a) the ability to respond to a challenge with SRBC in the hemolytic plaque assay and b) the ability to reject third party skin grafts, H-2 different to donor and recipient.

C. Material and Methods

Mice: C57BL/6, CBA, BALB/c and (C57BL/6×CBA) F_1 mice were obtained from Jackson Laboratory Bar Harbor Maine. The following short designations are used throughout this report: CBL, CBA, BALB and CBL/CBA. The mice were generally 8–12 weeks of age except where stated otherwise.

Chimeras: CBL → CBL/CBA or CBL/CBa → CBA were produced by a protocol already described previously [13]. The recipient mice were irradiated with 900 R (two opposite ^{137}cesium sources) and reconstituted one day later with 50×10^6 spleen or 20×10^6 bone marrow cells injected intravenously. These marrow cells had been pretreated with a specific xenogeneic anti-T-cell globulin (ATCG) [13] for 30 min at 4° C. In both chimera groups more than 90% of the transplanted animals survived. CBL → CBL/CBA chimeras were tested for chimerism by cytotoxicity with H-2 typing antisera. CBL/CBA → CBL chimeras were tested additionally by survival of CBA skin grafts.

The production of thymic chimeras was performed as follows. Recipient CBL/CBA mice 8 weeks of age were thymectomized. 14 days later the mice were transplanted with thymi of newborn CBL or CBA mouse donors. About 3–5 thymi per recipient were transferred unter the kidney capsule by a modified microsurgical technique originally described by East and Parrot [2]. Again 14 days later the recipients were irradiated and reconstituted with CBL or CBA T-cell deprived bone marrow as described above. Engraftment of the thymic implants was proven histologically in all mice after completion of the experiments.

Skin transplantation: Tail skin grafts were performed as described previously [2].

Hemolytic plaque assay: The immune response against SRBC was tested using a technique originally described by Jerne [5]. The chimeras were immunized each with 4×10^8 SRBC intraperitoneally 4 days prior to the test. For the evaluation of the secondary immune response the chimeras were boosted with the same number of SRBC 7 days after the first immunization and the test performed 4 days later. The hemolytic plaque test was performed as follows. 2×10^6 spleen cells and 40×10^6 SRBC were placed on agar beds in petri dishes and cultured for 1 h at 37° in an incubator. Direct hemolytic plaques (IgM response) were formed after addition of diluted ginea pig complement and further incubation at 37° C for 1 h. Indirect plaques (IgM + IgG response) were detected as described by Dresser and Wortis [1]. After 1 h incubation of the spleen cells with SRBC, diluted antimouse Ig was added for 1 h at 37° followed by addition of complement as already described. The number of plaque forming cells (PFC) was counted under indirect illumination in a dark field.

Immuno histochemistry: Tissue blocks of kidneys containing thymic grafts and spleens, lymph nodes and Peyer's patches were snap frozen in liquid nitrogen. Cryostat sections, 7 μm thick, were air dried and fixed in acetone for 5 minutes. The unlabeled antibody enzyme method originally described by Sternberger [15] and modified by Hoffmann-Fezer et al [4] was carried out as follows. T-lymphocytes: first incubation with ATCG produced in rabbits, second incubation with sheep-anti-rabbit immunglobulin in excess, third incubation with rabbit-peroxidase-anti-peroxidase (PAP) complex. B-lymphocytes: first step incubation by rabbit-anti-mouse-immunoglobulin, second and third steps incubations as for T-lymphocytes. Control serial sections were incubated at the first step with normal rabbit-immunoglobulin. Each incubation was done for 30 minutes at room temperatur and was finished by washing in Tris-buffered saline. Peroxidase activity was demonstrated with amino-ethyl-carbazol as described by Schaefer and Fischer [14]. Finally, sections were counterstained with hemalaun.

D. Results and Discussions

I. Reconstitution of Immune Responses in Parent-to-F1 Chimeras

The immune response of parent-to-F1 chimeras was tested using the following indicator system: CBL marrow cells were incubated with ATCG and transplanted to lethally irradiated CBL/CBA F1 hybrid recipients. The recipients were transplanted at 10 weeks of age (10-W) or at 70 weeks of age (70-W). The latter investigation was performed to determine whether the function of old involuted thymi may be reactivated by the bone marrow transplantation. Thymectomized and syngeneic transplanted recipients were used as controls. Thymectomized controls should ensure that any reconstitution of immune functions is impossible without thymus tissues, syngeneic transplanted controls should indicate the

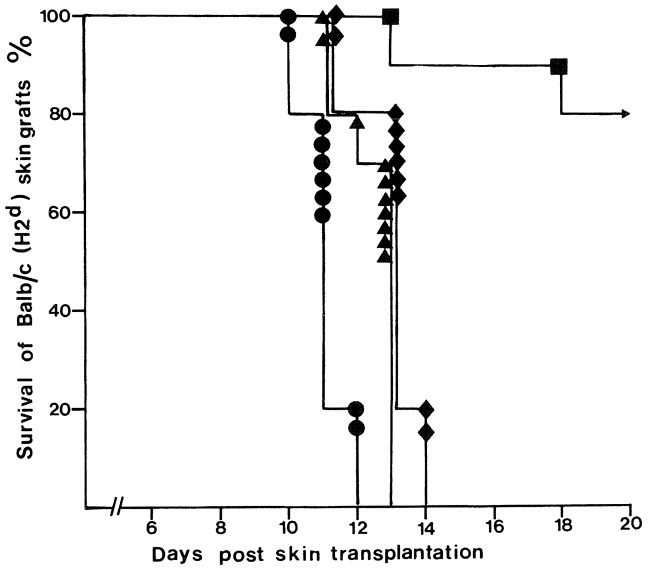


Fig. 1. Survival of third party BALB skin grafts on CBL → CBL/CBA chimeras transplanted with 20×10^6 T-cell-deprived bone marrow cells (■—■ thymectomized recipients, ▲—▲ recipients transplanted with 10 weeks of age, ◆—◆ recipients transplanted with 70 weeks of age, ●—● syngeneic transplanted control)

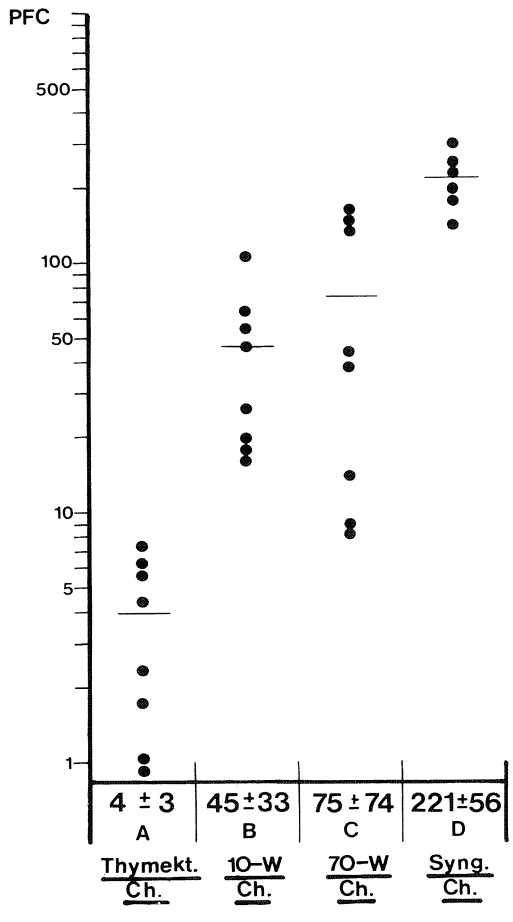


Fig. 2. Primary immune response against SRBC of CBL → CBL/CBA chimeras transplanted with 20×10^6 T-cell deprived bone marrow cells tested in the hemolytic plaque assay (A = thymectomized recipients, B = recipients transplanted at 10 weeks of age, C = recipients transplanted at 70 weeks of age, D = syngeneic transplanted control).

maximal immune response after bone marrow transfer without H-2 incompatibility. 60 days after transplantation the survival of BALB skin grafts, the response against SRBC and the repopulation of the lymphatic tissues with T-lymphocytes was determined.

Figure 1 shows the survival of third party BALB skin grafts on parent-to-F1 chimeras and controls. Syngeneic chimeras completely rejected their skin grafts before day 13. Most of the thymectomized controls on the other hand still carried intact skin grafts on day 20. Both groups of allogeneic parent-to-F1 chimeras, 10-W and 70-W recipients, showed a rejection of skin grafts not different from the syngeneic control group.

The immune response against SRBC was investigated in the hemolytic plaque assay. Figure 2 shows the primary direct response of parental-to-F1 chimeras as well as thymectomized and syngeneic transplanted control groups. The response of thymectomized chimeras was severely depressed (mean 4 PFC) compared to the 10-W as well as the 70-W chimeras showing significantly increased plaque counts (45 and 75 PFC). Syngeneic chimeras produced by transfer of CBL/CBA

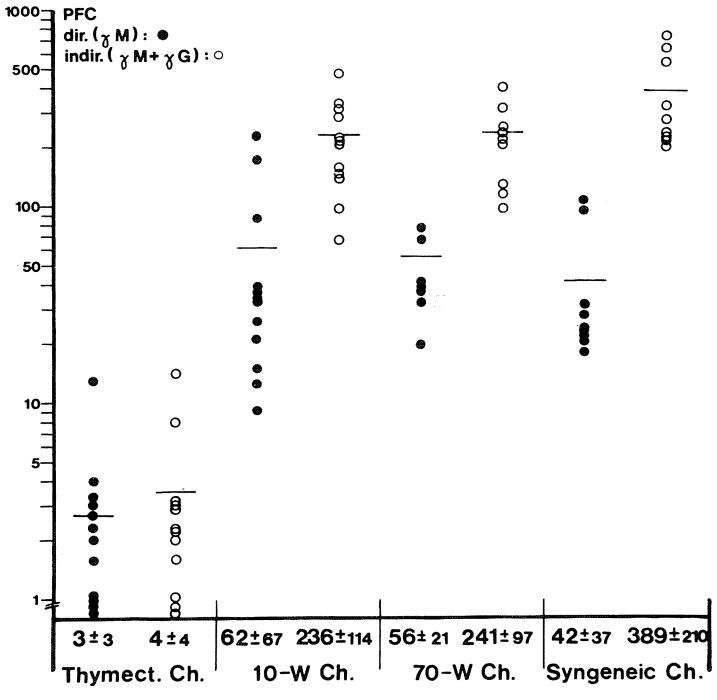


Fig. 3. Secondary direct and indirect immune response against SRBC of CBL → CBL/CBA chimeras transplanted with 20×10^6 T-cell-deprived bone marrow cells (from left to right: thymectomized recipients, recipients transplanted at 10 weeks of age, recipients transplanted at 70 weeks of age, syngeneic control)

marrow to lethally irradiated CBL/CBA recipients showed a still higher yield of plaques (221 PFC). This difference, however, can simply be explained by the fact, that CBL/CBA hybrids are higher primary responders against SRBC compared with the CBL strain. Syngeneic transplanted CBL-to-CBL controls showed PFC amounts within the range of those induced by parental-to-F1 chimeras (not shown here).

The secondary response against SRBC of similar chimera groups is presented in Fig. 3. Furthermore the conversion of 19s- to 7s-antibodies was analyzed by comparison of the direct (19s, dPFC) and the indirect (7s and 19s, iPFC) plaque formation. A second immunization did not result in any increase of the poor response in thymectomized chimeras (3 dPFC) nor was a conversion to the production of IgG-antibodies detectable (4 iPFC). In contrast the 10-W as well as the 70-W chimeras showed a marked increase of the direct plaque formation (62 PFC and 52 PFC) which was in the same range as the syngeneic control (42 dPFC). The high yields of indirect plaques in these groups (236 and 241 PFC) indicated a conversion to a considerable IgG antibody response comparable to the syngeneic control (389 iPFC). Parent-to-F1 chimeras produced by transplantation with T-cell-deprived spleen cells instead of bone marrow cells led to analogous reconstitution results.

In conclusion both test system did not detect a significant impairment of the immune response against incompatible skin grafts and SRBC in parent-to-F1 chimeras when compared with their syngeneic controls. These observations were confirmed by Sprent et al. [15] who investigated the immunoreconstitution in parent-to-F1 chimeras after transfer of bone marrow incubated with allogeneic anti-theta-serum. The results may further indicate that prevention of GVHD as achieved here through incubation treatment with ATCG will allow a prompt recovery of the immunologic functions.

Thymic involution and a decrease of immune reactivity have been observed in old mice [3] like in other species. Our recipients of one group that were 70 weeks of age, showed a reduction of their thymic tissue mass to about 1/3 of the weight of 8 weeks old mice. This fact, however, did not appear to play a major role for the reconstitution of the immune response in the transplanted animals. 10-W chimeras responded equally well as 70-W chimeras to incompatible skin grafts and SRBC. When thymi of the old age involuted mice where inspected 60 days after marrow transplantation, the thymic tissue had been reconstituted to the weight of young mice and was fully repopulated with donor type T-lymphocytes.

Figure 4 compares the gradual development of the immune response against SRBC at different times of the post transplant phase. Both syngeneic and parent-to-F1 chimeras reached their full capability to respond to a challenge with SRBC between day 60 and day 80 after bone marrow transfer. The extend of the response after complete reconstitution varied with the donor strain and was not different from the response of untreated animals of the respective strain.

The anatomical repopulation of the lymphatic organs with T-lymphocytes was investigated by immunohistochemistry using ACTG combined with the PAP technique. T-cells were visible by a brown reaction product on the cell membrane.

Table 1. Repopulation of the lymphatic tissues in CBL → CBL/CBA chimeras after lethal irradiation and transfer of 20×10^6 T-cell-deprived bone marrow cells, investigated with the PAP method

Group	Thymus[a]	Splenic white pulp[b]		Lymph node[c]	Peyer's patches[d]
		periarteriolar	peripheral		
CBL → CBL/CBA, thymectomized	−	(+)/−	+++	(+)/−	(+)/−
CBL → CBL/CBA, 10 W – recipients	+++	+++	+++	++	++
CBL → CBL/CBA, 70 W – recipients	+++	++	+++	++	++
CBL/CBA → CBL/CBA syngeneic control	+++	+++	+++	+++	+++

Repopulation of

[a] the thymic cortex with T-lymphocytes

[b] the periarteriolar lymphatic sheaths with T-lymphocytes and the peripheral white pulp with B-lymphocytes

[c] the paracortical area with T-lymphocytes

[d] the interfollicular areas with T-lymphocytes

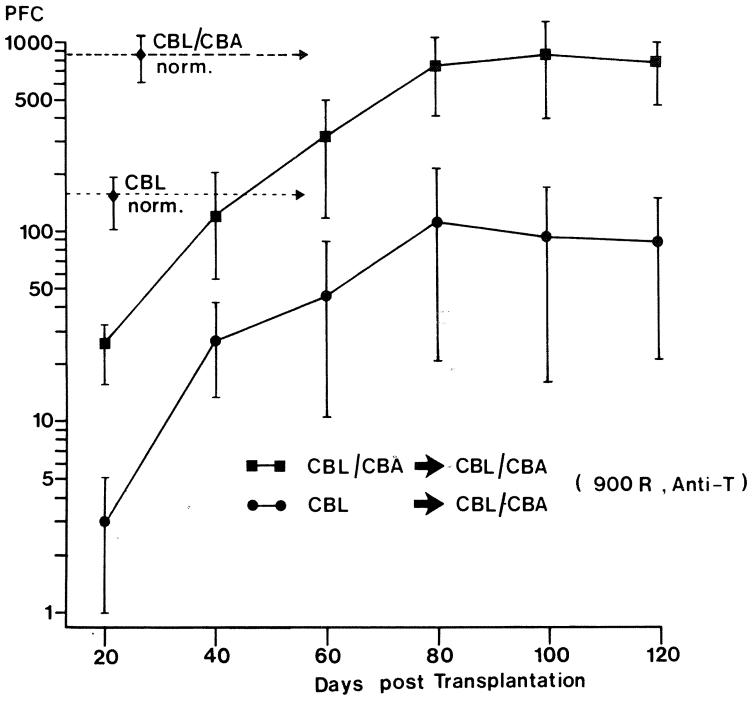


Fig. 4. Reconstitution of the primary immune response against SRBC of CBL → CBL/CBA chimeras and syngeneic transplanted controls after various days post transplantation

The results are shown in Table 1. Thymi of old and young chimeras as well as the syngeneic controls are well repopulated by thymic lymphocytes. Periarteriolar lymphatic sheaths of the splenic white pulp, paracortical areas of the lymph nodes and interfollicular zones of Peyer's patches were repopulated by T-lymphocytes with somewhat reduced density in the following sequence: syngeneic transplanted recipients followed by 10-W parent-to-F1 chimeras followed by 70-W parent-to-F1 chimeras. A very scanty occurence of singular T-lymphocytes only can be observed in thymectomized mice. B-lymphocytes were distributed equally dense in the peripheral parts of spleen white pulps, lymph node cortex and follicles of Peyer's patches of all the groups including the thymectomized chimeras. Typing of the lymphocytes of the lymphatic organs with anti-H-2 antisera revealed only donor type cells.

The anatomical repopulation corresponds well with the former reported functional results. In parent-to-F1 chimeras the recipient thymus obviously matured stem- and/or progenitor cells from T-cell-deprived donor marrow to immunocompetent T-lymphocytes. Involuted thymi in old recipients were reactivated after marrow transplantation, so that the old recipients respond similar to recipients transplanted before thymic involution. Matured T-lymphocytes repopulated the lymphatic organs and were able to respond to H-2 incompatible skin grafts and SRBC. After sufficient elimination of T-lymphocytes from the graft the genetical one way H-2 incompatibility against the host had therefore no effect on the immunological reconstitution.

II. Reconstitution of Immune Responses in F1-to-Parent Chimeras

The immune response of F1-to-parent chimeras was analyzed using the following indicator system: CBL/CBA marrow cells were incubated with ATCG and transplanted to lethally irradiated CBA recipients. The chimerism of the recipients was proven by permanent survival of CBL skin grafts prior to the experiments. As already described for the parent-to-F1 chimeras thymectomized chimeras and recipients transplanted with syngeneic marrow served as control. 60 days after transplantation the survival of BALB skin grafts and the response against SRBC was determined.

After transplantation of third party BALB skin grafts to the chimeras from the different groups the following results were observed: F1-to-parent chimeras rejected the skin grafts in about the same time as syngeneic transplanted controls. The survival of BALB skin in thymectomized chimeras on the other hand was almost not affected. Therefore skin graft survival data did not differ from those obtained in the parent-to-F1 model.

The immune response of the described chimera groups against SRBC is indicated in Fig. 5. The primary response of the thymectomized control group is almost completely reduced (6 PFC). F1-to-parent chimeras showed a reconstituted immune response by a significantly increased number of plaques (84 PFC), although the number of plaques in syngeneic transplanted CBL/CBA-to-CBL/CBA control group exceeded somewhat that of the former group (240 PFC). The reason for this difference is presently not completely understood, since in both

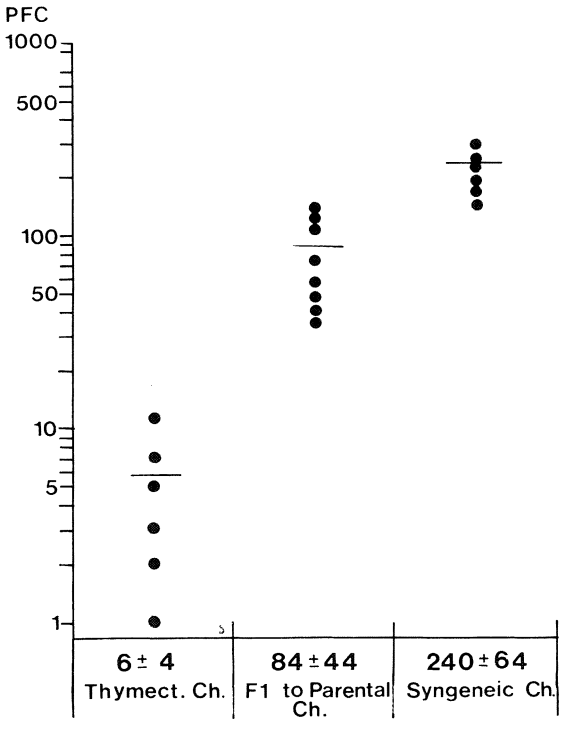


Fig. 5. Primary immune response against SRBC of CBL/CBA → CBA chimeras transplanted with 20×10^6 T-cell-deprived bone marrow cells tested in the hemolytic plaque assay (from left to right: thymectomized recipients, normal recipients, syngeneic transplanted control)

groups the same mouse donor strain was used for production of the chimeras. Certainly some anti-graft-reactivity cannot be excluded. Although recent data have indicated that the major histocompatibility complex (MHC) influences the T-cell responsiveness and that T-lymphocytes require the presence of MHC-identical thymus for proper antigen processing, these phenomenons seem not to be responsible for the above described results [21] as also shown in the following paragraph.

III. Reconstitution of Allogeneic Thymus Chimeras

The immune response of allogeneic thymus chimeras was investigated in the following indicator system: CBL/CBA recipients were thymectomized and transplanted with CBA-thymus tissue. After lethal irradiation these animals were reconstituted with T-cell-deprived CBL marrow cells (group 1). Thymectomized recipients without thymus transplantation (group 2) and CBL/CBA recipients with syngeneity between transplanted thymus and transferred bone marrow cells (group 3 and 4) served as controls. All different groups are indicated in Table 2.

Table 2. Influence of a complete H-2 difference between transplanted thymus tissue and T-cell deprived bone marrow cells on the immune response of CBL/CBA recipient mice against SRCB and BALB skin grafts

Group	Transplanted		Rejection of BALB skin grafts[b] (days)	Response against SRBC[c]		
	Thymus[a] (H-2)	Bone marrow[a] (H-2)		Primary, direct	Secondary, direct	Secondary, indirect
1	CBA (k)	CBL (b)	17	131±90[d]	711±284	1110±306
2	– No –	CBL (b)	>49	9± 5	20± 18	29± 20
3	CBL (b)	CBL (b)	17	56± 24	601±304	893±403
4	CBA (k)	CBA (k)	16	413±201	389±189	1178±498

[a] Recipients = CBL/CBA hybrids, transplantation protocol see material and methods
[b] Mean rejection time of BALB (H-2^d) skin grafts, H-2 different to thymus and bone marrow cells
[c] Number of plaque-forming cells (PFC)/2×10^6 spleen cells
[d] PFC ± S.D.

60 days after bone marrow transplantation the allogeneic thymus chimeras were investigated for their ability to respond against SRBC and third party BALB skin grafts in comparison to the controls. The anatomical repopulation of the lymphoid tissues with T-lymphocytes was analyzed by immunohistochemistry.

All chimera groups were investigated for sufficient engraftment of the thymic transplants. Thymi grafted under the kidney capsule in the allogeneic group 1 were well divided into thymic cortex and medulla. Both compartments were densely repopulated with T-lymphocytes. An example of a thymic graft in one of the allogeneic thymus chimeras is given in Fig. 6. No difference in grade of engraftment and repopulation was seen when compared with syngeneic groups 3 and 4.

The ability of the allogeneic thymus chimeras to reject third party BALB-skin grafts is shown in Table 2. Thymectomized recipients without thymus transplanta-

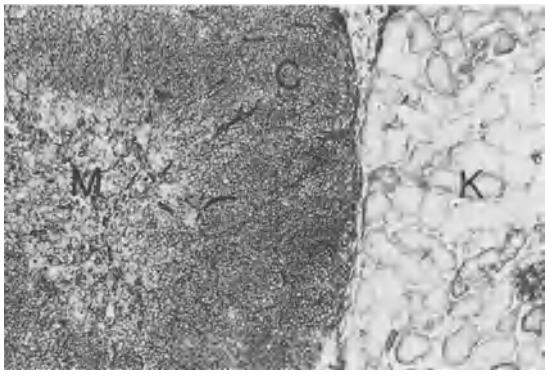


Fig. 6. Well developed CBA thymic graft under the kidney capsule of a CBL/CBA mouse, C=thymic cortex, M=thymic medulla, K=cortex of kidney, ATCG, ×80

tion (Group 2) still carried skin grafts longer than 49 days. Chimeras with syngeneity between grafted thymus and transferred marrow (group 3 and 4) rejected skin grafts until day 16 to 17 after marrow transplantation. The allogeneic chimeras with CBA thymus and CBL bone marrow (group 1) were able to reject the BALB skin in the same time (17 days) as the syngeneic controls.

The immune response of the same chimera groups against SRBC is also indicated in Table 2. Chimeras without thymus (group 2) do almost not respond against SRBC (primary response PFC, secondary response 20 dPFC, 29 iPFC). In contrast, allogeneic thymus chimeras (group 2) showed a marked increase of their response against SRBC (primary response 131 PFC, secondary response 711 dPFC, 1110 iPFC) and a conversion of IGM to IgG response in about the same range seen in the syngeneic control groups 3 and 4.

The investigation of the anatomical repopulation of the lymphatic tissues with T-lymphocytes revealed the following results: groups 1, 3 and 4 having received a thymic graft had periarteriolar lymphatic sheaths in the spleen and paracortices in the lymphnodes richly repopulated by T-lymphocytes without visible differences between the allogeneic and the syngeneic groups (Fig. 7a, b), whereas in animals without thymic graft only very few singular T-positive lymphocytes were distributed in the T-cell zones of spleens (Fig. 7c) and lymph nodes. B-cell zones, i.e. peripheral parts of spleen white pulp and lymphnode cortices were densely populated by Ig-positive lymphocytes in groups with as well as without a thymic graft.

The results in skin graft rejection, SRBC response and T-cell repopulation confirmed that – inspite of a complete H-2 allogeneity between thymus and T-cell-deprived donor marrow – the recipient thymus matured H-2 different stem and/or progenitor cells to immuno-competent T-lymphocytes. These T-lymphocytes repopulated the lymphatic organs and were able to respond to histocompatibility – and foreign antigens so far investigated. These observations were unexpected, because already earlier reports have shown that differences at the major histocompatibility locus between stem cells and thymus may lead to an impaired immune function in the transplanted animals [18, 6, 8]. Recent reports of Zinkernagel [21] showed that certain T-cell functions required MHC syngeneity of thymus and maturing T-lymphocytes. On the other hand, Wagner et

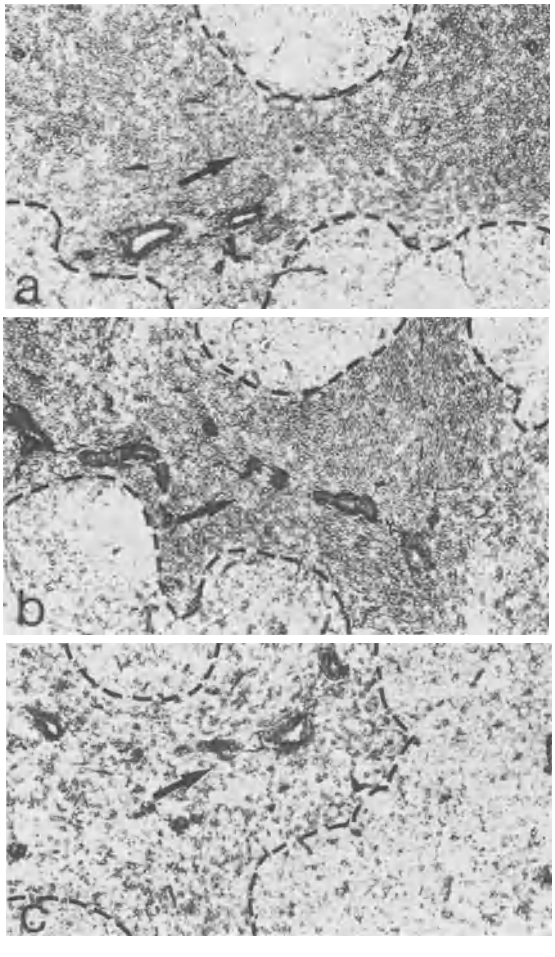


Fig. 7. Periarteriolar lymphatic sheaths of spleen white pulp (encircled by black lines) **a** densely populated by T-lymphocytes (↑) in a CBL/CBA mouse grafted with CBA thymus and CBA bone marrow (group 4), **b** T-cell (↑) repopulation of equal density in a CBL/CBA mouse grafted with CBA thymus and CBL bone marrow (group 1) **c** loosely settled by T-lymphocytes (↑) in a CBL/CBA mouse without thymic graft but grafted with CBL bone marrow (group 2), ATCG, ×80

al. (this issue) showed that these fully allogeneic chimeras were able to mount both alloreactive cytotoxic T lymphocytes (CTL) as well as H-2 restricted virus specific CTL. Although these results have to be confirmed in mouse strain combinations with other H-2 haplotypes, the data indicate that bone marrow transplantation across the MHC-barrier does not prevent the restauration of immunocompetence against H-2 and foreign antigens.

Acknowledgements

This study was supported by GSF-EURATOM 217-76-1 BIOD and SFB 37/E3.

We thank Ms. B. Dellekönig, Ms. B. Glöckner, Ms. J. Molitor and Mrs. S. Zimm for their skillfull technical assistance.

References

1. Dresser, D. W., Wortis, H. H.: Use of antiglobulin serum to detect cells producing antibody with low hemolytic efficiency. Nature *208,* 859–861 (1965)
2. East, J., Parrott, D.: Prevention of wasting in mice thymectomized at birth and their subsequent rejection of allogeneic leukemic grafts. J. Nat. Cancer Inst. *33,* 673 (1964)
3. Hirokawa, K., Makinodan, T.: Thymic involution: Effect on T-cell differentiation. J. Immunol. *114,* 1659–1664 (1975)
4. Hoffmann-Fezer, G., Rodt, H., Eulitz, M., Thierfelder, S.: Immunohistochemical identification of T- and B-lymphocytes delineated by the unlabeled antibody enzyme method. I. Anatomical distribution of D-positive and Ig-positive cells in lymphoid organs of mice. J. Immunol. Meth. *13,* 261–270 (1976)
5. Jerne, N. K., Nordin, A. A.: Plaque formation in agar by single antibody-producing cells. Science *140,* 405 (1963)
6. Kindred, B., Loor, F.: Activity of host-derived T-cells which differentiate in nude mice grafted with co-isogeneic or allogeneic thymuses. J. Exp. Med. *139,* 1215–1227 (1974)
7. Kolb, H. J., Rieder, I., Rodt, H., Netzel, B., Grosse-Wilde, H., Scholz, S., Schäffer, E., Kolb, H., Thierfelder, S.: Antilymphocytic antibody and marrow transplantation VI. Graft-versus-host tolerance in DLA-incompatible dogs following "in vitro" treatment of bone marrow with absorbed antithymocyte globulin. Transplantation (in press)
8. Loughman, B. E., Nordin, A. A., Bealmar, P. M.: Studies of the immunological capacity of germ-free mouse radiation chimeras. I. Chimerism and humoral immune response. Cell. Immunol. *9,* 104–117 (1973)
9. Lydyard, P. M., Ivanyi, J.: Chimerism of immunocompetent cells in allogeneic bone marrow-reconstituted lethally irradiated chickens. Transplantation *20,* 155–162 (1975)
10. Rodt, H.: Herstellung und Spezifität heterologer Anti-T-Lymphozyten-Seren und ihre Anwendung im Rahmen der Knochenmarktransplantation und der Leukämiediagnostik. GSF Bericht H 604, München 1979
11. Rodt, H., Thierfelder, S., Eulitz, M.: Suppression of acute secondary disease by heterologous anti-brain-serum. Blut *25,* 385–389 (1972)
12. Rodt, H., Thierfelder, S., Eulitz, M.: Antilymphocytic antibodies and marrow transplantation. III. Effect of heterologous anti-brain antibodies on acute secondary disease in mice. Eur. J. Immunol. *4,* 25–29 (1974)
13. Rodt, H., Kolb, H. J., Netzel, B., Rieder, I., Janka, G., Belohradsky, B., Haas, R. J., Thierfelder, S.: GVHD suppression by incubation of bone marrow grafts with anti-T-cell globulin: Effect in the canine model and application to clinical bone marrow transplantation. Transpl. Proc. (in press)
14. Schaefer, H. E., Fischer, R.: Der Peroxydase-Nachweis an Ausstrichpräparaten sowie an Gewebeschnitten nach Entkalkung und Paraffineinbettung. Klin. Wochenschr. *46,* 1228–1230 (1968)
15. Sprent, J., v. Böhmer, H., Nabholz, M.: Association of immunity and tolerance to host H-2 determinants in irradiated F1-hybrid mice reconstituted with bone marrow cells from one parental strain. J. Exp. Med. *142,* 321–331 (1975)
16. Sternberger, L. A.: Immunocytochemistry, pp. 129–137. Englewood Cliffs, N. J.: Prentice Hall Inc. 1974
17. Trentin, J. J., Judd, K. P.: Prevention of acute graft-versus-host (GVH) mortality with spleen-absorbed anti-thymocyte globulin (ATG). Transpl. Proc. *5,* 865–868 (1973)
18. Urso, P., Gengozian, N.: T-cell deficiency in mouse allogenic radiation chimeras. J. Immunol. *111,* 712–719 (1973)
19. Urso, P., Gengozian, N.: Variation in T- and B-cell deficiency in different mouse allogeneic radiation chimeras. J. Immunol. *113,* 1770–1778 (1974)
20. Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, J., Klein, P. A., Klein, J.: On the thymus in the differentiation of "H-2 self-recognition" by T-cells: Evidence for dual recognition. J. Exp. Med. *147,* 882–896 (1978)

Discussion

van Bekkum: Could you say that the immune response of F1-into-parent chimeras is not much different from the syngeneic control?
Rodt: This is correct provided you use the same donor strain.
Waldman: I feel I should say something because we presented differing data this morning. The story of allogeneic chimeras is an interesting one because with strong antigens we also have got an allogeneic response to KLH. The question is, can we consider allogeneic chimeras fully immunocompetent to all antigens. If they have a limited number of determinants, for instance Ir gene antigens, you may get a different picture.
Riethmüller: In the animals where you grafted the CBA thymus under the kidney capsule and injected the CBl cells whether they are cytotoxic in an allogeneic restricted fashion?
Rodt: This was done by Dr. Wagner who used the same chimeras.
Wagner: I think I have to comment after Herman Waldman's comment. The question was whether the same chimeras are immunocompetent to so-called weak antigens. The data I presented this morning came from the same batch of mice. The same allogeneic H-2 incompatible chimeras were able to make H-2 restricted sendai virus specific ctl, so the conclusion from the data would suggest that immunocompetence to a variety of different antigens on both the T-B collaboration level and the T cytotoxic level is existent.
Kersey: Are the data of the syngeneic controls comparable to normal mice? Because in the studies of others normal mice were always better than syngeneic chimeras whose immunocompetence always used to be subnormal.
Rodt: The syngeneic controls did not differ from normal mice. But you must keep in mind that our chimeras were tested long after transplantation, between 60–80 days. At that time immune reconstitution had completely recovered.

Surface Antigens Characterizing Lymphopoietic Stem Cells in Rats

S. V. Hunt

A. Introduction

Until very recently, the only clue to the stage of development of a particular cell type during hemopoietic differentiation has been its morphology. All the classical studies on the differentiation of erythroid and granulocytic cells, for example, used the staining properties and the sizes of cells in smears to work out the stages of development to mature erythrocytes and granulocytes. However, in the case of lymphocyte differentiation this approach is less rewarding since lymphocytes are relatively featureless cells. Mature T and B lymphocytes, which are very different in their functions and ontogeny, cannot be told apart simply by smearing and microscopic examination. An alternative approach pioneered largely in experiments with mice is to look for stage-specific differentiation antigens of the cell surface and follow their appearance and disappearance during ontogeny [1]. This should have three advantages. It should allow more precise staging of development, with interrelationships between cell types becoming clearer, especially if several antigenic markers can be considered together. It should allow the cells to be manipulated experimentally by employing the markers in purification techniques. Finally it may ultimately be possible to relate the molecule bearing the differentiation antigen to a particular function that it performs for the cell. The advent of hybridoma antibodies to define antigens clearly [2, 3] has given a particular impetus to this line of attack on the problem of lymphopoiesis.

This paper describes assays for rat T and B lymphopoietic stem cells, outlines the tissue distribution of the stem cells and reports initial studies with some surface antigens (Thy-1, surface immunoglobulin and Ia-like) that may help to characterize early stem cells.

B. Methods

I. Assays for Lymphopoiesis

Micklem and his colleagues [4] long ago showed that it was possible to construct stable mouse radiation chimeras where donor hemopoietic cells which were distinguished by a chromosome marker visible at metaphase competed with a standard inoculum of bone marrow cells so that stem cells could "race" against each other in irradiated hosts. They analyzed the spontaneous or mitogen-indu-

ced mitoses of various lymphoid tissues of the chimeras for the proportions of cells derived from the "test" and "standard" inocula. The assays to be outlined here (a full account is in preparation) use the same principle of competition with a reference inoculum but different markers. These markers avoid restricting the analysis to cells only in mitosis and define clearly the cell type (B or T lymphocyte) under study. For the assay of B stem cells an allotype of the light chain of rat immunoglobulins [5] was employed, while the alloantigenic system Pta (peripheral T cell alloantigen) [6, 7] is suitable for T cells. The immunoglobulin marker accounts for virtually all immunoglobulin-bearing cells since the allotypy is of kappa chains which constitute more than 95% of adult immunoglobulins [8]. The Pta marker accounts for most but not quite all T cells in the periphery; between 10 and 20% of rat thoracic duct lymphocytes carry neither surface immunoglobulin nor Pta [7, 9]. Rats bearing the markers have been bred separately by repeated backcrossing to give partner strains congenic with the PVG strain used as irradiated host and provider of reference bone marrow. Figure 1 summarizes the experimental protocol. It will be noted that the analysis of chimerism is performed on thoracic duct lymphocytes since they provide the purest source of fully mature B and T lymphocytes.

II. Antisera [10]

1. For Chimera Analysis

To detect all thoracic duct B cells regardless of allotype, immunoadsorbent purified rabbit $(Fab')_2$ anti rat Fab was used, its binding being revealed by

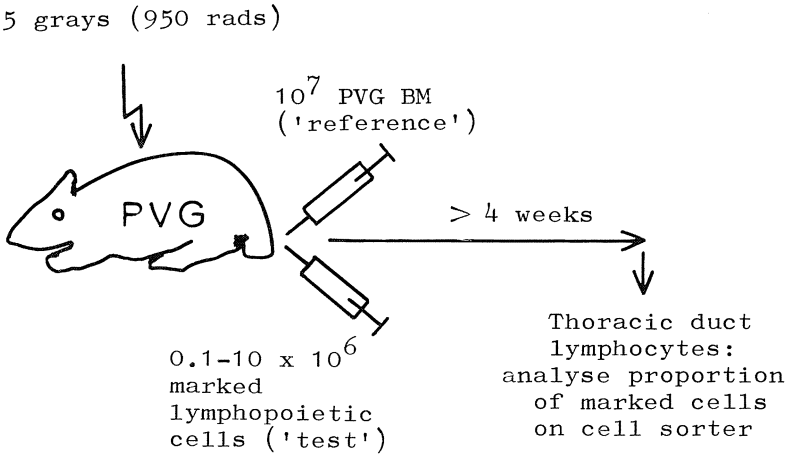


Fig. 1. Protocol for lymphopoietic assays. Donor cells carry the appropriate genetic marker (Ig-1a for B cell assay, Pta_{DA} for T cell assay) and are congenic with the recipient. These cells may either be unseparated cells from various lymphoid tissues or they may be injected after labeling with antibody to surface markers and separation on a fluorescence-activated cell sorter. They compete with a fixed dose of unmarked young adult bone marrow cells in irradiated PVG hosts. With unseparated marrow as the "test" inoculum the chimerism eventually measured amongst T or B lymphocytes in the thoracic duct reflects the proportions of the two kinds of marrow injected. This chimerism is stable for at least one year.

a second layer of fluorescein-conjugated horse $(Fab')_2$ anti rabbit IgG. Specificity was controlled by inhibition of the first layer with purified rat IgG. Background staining was 0.4%.

Immunoglobulin allotype-bearing cells were revealed by incubation with immunoadsorbent-purified fluorescein-conjugated rat IgG anti-Ig-1a allotype, specificity again being controlled by inhibition with IgG of the Ig-1a allotype. Background staining was 0.17%.

Anti-Pta serum was raised by immunising PVG rats with AUO spleen and lymph node cells [6]. IgG from this was used at 4 mg ml^{-1} and its binding was revealed by a second layer of immunoadsorbent-purified fluorescein-conjugated rabbit $(Fab')_2$ anti-rat γ_2. Background staining (on PVG thoracic duct lymphocytes) was 1.5%.

2. Other Antisera

Anti-Thy-1 sera were of two kinds: fluorescein-conjugated rabbit $(Fab')_2$ anti-rat brain Thy-1 [11–13] whose specificity was controlled by inhibition with purified rat thymocyte Thy-1 L+ antigen [14]. Background staining on nucleated bone marrow cells was 1.8%. Alternatively, C3H mouse anti-AKR mouse thymocyte serum (1 in 50) followed by a second layer of fluorescein-conjugated rabbit $(Fab')_2$ anti-mouse Ig was used, specificity being controlled by comparing absorption with A/Jax or A-Thy-1.1 brain homogenate. Background staining on nucleated bone marrow cells was 1.9%.

Anti-Ia antisera were from two sources; a monoclonal mouse anti-rat purified Ia antibody (MRC OX 4, [15]) and a serum raised in (PVG-RT-$1^u \times$DA) F_1 rats against PVG-RT-1^{u1} spleen and lymph node cells [7]. Their binding was revealed by fluorescein-conjugated $(Fab')_2$ anti-mouse or anti-rat Ig respectively. These antibodies labeled about 8% of bone marrow nucleated cells with a background labeling of 1.5%.

III. Cell Sorting

A FACS-II (Becton Dickinson) was used to separate labeled from unlabeled bone marrow or fetal liver cells. The purities of the fractions were checked in each experiment and for the negative cells were 97 to 99%. The purity of the labeled cells depended on the initial frequency of positive cells before sorting but was usually between 95 and 99%. Total cell recoveries were between 30 and 40%.

In experiments studying surface Thy-1 control cells were always run in which the incubation had been blocked with specific antigen (see Sect. B II.2) but were otherwise treated identically to labeled cells. These "sorted" control cells were tested for lymphoid stem cell function. This was especially important for fetal liver since the fluorescence intensity profile declined monotonically [9] and was not greatly above background so that the positioning of the thresholds for sorting was arbitrary.

IV. Animals

Donors were either young adult male rats, 8 to 14 weeks, or fetal rats at 17–20 days of gestation. They were of the PVG-1a and PVG-Pta_{DA} strains, which are

the genetically marked partner strains congenic with the young adult PVG rats that were used as hosts.

Table 1. Tissue distribution of T and B lymphoid stem cells

B stem cells						
Tissue	Cell dose $\times 10^{-6}$	Percent chimerism Mean ± S.E.	No. rats	Cell dose $\times 10^{-6}$	Percent chimerism Mean ± S.E.	No. rats
Young adult (8–11 wks)				*Suckling (14 days)*		
Bone marrow	10	29.5[a] ±4.1	9	10	78.3[a] ±0.7	3
Spleen	10	8.3 ±1.3	9	10	89.8 ±2.8	7
Thoracic duct lymphocytes	10	7.7 ±1.8	12		N.D.	
Lymph node	10	3.1 ±0.7	7		N.D.	
Peyer's patch	7	5.3 ±2.4	2	–		
Peyer's patch	4	0.85 ±0.2	3	1.5–3	0.3 ±0.2	2
Thymus	100	0.25 ±0.1	4	–		
Thymus	10	0.3 ±0.1	7	10	0.2 ±0.08	5
Fetus (17–20 day's gestation)						
Spleen	1.0	49.0[a] ±4.8	2			
Spleen	0.3	26.4 ±4.5	2			
Liver	4.3	79.1 ±2.1	13			
Liver	1.1	53.7 ±3.4	8			
Liver	0.3	35.8 ±5.2	8			
Liver	0.1	24.0 ±4.1	10			
T stem Cells						
Young adult (8–14 wks)						
Bone marrow	10	33.6[b] ±3.6	7			
Bone marrow	4.3	16.7 ±0.6	6			
Bone marrow	1.1	9.5 ±1.8	7			
Fetus (17–20 day's gestation)						
Liver	4.3	49.0[b] ±7.5	6			
Liver	1.1	30.9 ±5.3	4			
Liver	0.3–0.5	11.7 ±3.4	6			
Spleen	0.7–1.1	31.3 ±5.2	3			
Spleen	0.25	23.1 ±7.1	3			
Thymus	8	0.4 ±0.2	2			
Thymus	0.8	1.5 ±0.6	3			

[a] Frequency of Ig-1a marked cells in TDL divided by frequency of surface immunoglobulin positive cells

[b] Frequency of Pta_{DA} marked cells amongst TDL depleted of B cells, by rosetting with anti-Fab coated erythrocytes and separation on Isopaque-Ficoll

C. Results

I. Tissue Distribution of Lymphoid Stem Cells

Cell suspensions from a variety of adult and fetal lymphoid tissues were competed against the reference dose of 10^7 bone marrow cells and the chimerisms of the thoracic duct T and B lymphocytes were measured in the irradiated hosts four weeks or more after reconstitution. Table 1 shows the following. First, in the young adult the potency of B lymphoid activity decreases in the order Marrow>Spleen>Lymph Nodes=Peyer's patch=Thoracic duct lymphocytes. The activity of the marrow and of the spleen was greater in pre-weanling rats and greatest in the liver and spleen of near-term fetuses; indeed, in these recipients of younger tissues there was a tendency (data not shown) for the test cells to overgrow the reference marrow inoculum with increasing time after reconstitution. This suggests a qualitative difference in the self-renewal or proliferative capacities of the cells from younger donors. A second point to be made from Table 1 concerns the inertness of thymocytes. Neither in the B assay using adult thymus nor in the T assay using fetal thymus, even with large doses of cells, could any mature progeny be detected. Provided that this is not merely an artefact of a possible peculiar susceptibility of thymocytes to trauma, these results mean that the assays measure a cell type with considerable proliferative potential which has yet to enter the thymus and can overgrow thymocytes despite the latter's undoubted capacity to manufacture T cells.

The balance between test and reference cells in the chimeras will depend on their relative abilities to renew themselves and to generate differentiated progeny. These progeny may in their turn have substantial self-renewal capacity. It may therefore not be a single cell type but a complicated mixture early in the differentiation pathway that is measured in this chimerism assay. Abramson et al. [16] have found evidence for a variety of degrees of restriction of differentiation potential in early lymphopoiesis in the mouse. The unravelling of any early heterogeneity there may be will have to be done by the use of surface antigens and by the application of improved assays.

II. Thy-1 Antigen on Lymphopoietic Stem Cells

The observations that many rat bone marrow cells carry the Thy-1 antigen [17] and that some of these (about one fifth) also carry surface immunoglobulin [10] suggested that this antigen might be involved in B cell development. Hence in separate experiments bone marrow and fetal liver were separated by a fluorescence activated cell sorter into Thy-1 positive and negative fractions for testing their B lymphopoietic activity. The results (Table 2) showed all the B lymphoid stem cells in both tissues to be Thy-1 positive. Furthermore the chimeras were stable. The proportion of test cells did not wane even nine months or more after reconstitution, nor did any significant B lymphopoietic activity ever appear from the Thy-1 negative fraction. This stability re-emphasizes the notion suggested earlier (Sect. C I.) that the assay measures an early, self-renewing cell type.

Table 2. B lymphopoietic activity of sorted Thy-1 positive and Thy-1 negative bone marrow and fetal liver

Source	Cells	Dose ($\times 10^{-6}$)	No. of rats	% Ig-1a amongst thoracic duct B lymphocytes mean ± S.E.
Bone marrow	Labeled, unsorted	4.3	4	26.3 ± 10.4
	Labeled, unsorted	1.1 – 2	9	10.1 ± 1.7
	Thy-1$^+$	1.1 – 2	11	14.1 ± 4.2
	Thy-1$^-$	2	12	1.3 ± 0.6
Fetal liver	Incubated with inhibited anti-Thy-1 and sham sorted	4.3	5	77.8 ± 4.6
		1.1	3	66.1 ± 4.3
	Thy-1$^+$	0.3	3	67.3 ± 8.1
	Thy-1$^+$	0.1	7	58.5 ± 3.5
	Thy-1$^-$	4.3	7	6.2 ± 3.2
	Thy-1$^-$	1.1	5	3.5 ± 2.1

Similar experiments were then performed looking for Thy-1 on T lymphoid stem cells. Again, essentially all T lymphopoietic activity of both bone marrow and fetal liver was in the Thy-1 positive fraction and Thy-1 negative cells showed no significant activity even in long-term chimeras. In both T and B lymphopoietic studies the results were no different whether xeno-anti-Thy-1 made in rabbits or mouse allo-anti-Thy-1.1 was used for labeling. Finally, Thy-1 was sought on CFU_s again by sorting bone marrow cells following labeling with $(Fab')_2$ xeno-antibody. Although in rats we have had difficulty in obtaining reliable spleen colonies the results were clear that CFU_s carried Thy-1 (data not shown).

III. Other Surface Antigens

Mature B lymphocytes carry substantial amounts of surface immunoglobulin ($2–10 \times 10^4$ molecules per cell [18]) and surface Ia antigens. To test how far back these markers could be found during B lymphopoiesis, bone marrow cells were labeled with appropriate antisera and sorted for test in the long-term chimerism assay. No activity was found either amongst surface immunoglobulin positive cells [10] or Ia positive cells (data not shown). Both these antigens therefore appear during maturation and are not present initially.

IV. Migration Studies

A currently popular hypothesis concerning mammalian B cell development proposes that the role of Bursa of Fabricius in birds is performed by fetal liver and bone marrow themselves and that cells emigrate from these sites in a partly or fully mature state [19]. If this is correct, it would be interesting to discover whether emigration precedes or follows the appearance of surface immunoglobu-

lin and the disappearance of Thy-1. If one could isolate from marrow immature cells carrying Thy-1 but not immunoglobulin, it would be possible to test this and then proceed to a formal demonstration that the cells later acquired immunoglobulin. Therefore preliminary studies have been undertaken to study the fate of ^{51}Cr-labeled "Thy-1 only" marrow cells when transferred intravenously to syngeneic recipients. These cells were prepared by depleting marrow by a rosetting procedure of cells carrying immunoglobulin or the antigens defined by the monoclonal antibodies W3/13 (mainly polymorphs) and W3/15 (mainly erythrocytes) [3]: the residue contains about 90% Thy-1 positive cells. A typical experiment which examined the distribution of chromium label 20 hours after transfusion showed a substantial homing back to the marrow, with some localisation also in liver and spleen (Table 3). Essentially none remained in the blood nor went to the thymus or small intestine. Though the cells were of good viability (>90% by trypan blue exclusion), it is not yet clear whether the liver localisation is an artefact of the trauma of the treatment of the cells or a physiological tendency to home there. The data do, however, suggest that some cells bearing Thy-1 may migrate to the spleen before acquisition of surface immunoglobulin and the overall tissue distribution confirms the findings of Yoshida and Osmond [20] who followed the migration of recently-divided bone marrow cells labeled in situ with tritiated thymidine.

	% of recovered label (cpm)
Marrow, limbs	25.4
Marrow, rest of skeleton	29.1
Liver	24.6
Spleen	17.2
Lung	2.2
Kidneys	0.7
Blood (6 ml)	0.3
Small intestine	0.2
Mesenteric lymph node	0.07
Thymus	0.06
Cervical lymph nodes	0.03
	99.9

Table 3. Localization of marrow Thy-1$^+$ sIg$^-$W3/13$^-$W3/15$^-$ cells 20 hours after intravenous injection. Femoral+tibial marrow from a 4-week old donor was labelled with Na_2 $^{51}CrO_4$ (20 μCi/ml) by a 60 minute incubation in RPMI 1640/10% fetal calf serum. Cells carrying surface immunoglobulin, W3/13 and W3/15 antigens were removed by a rosetting procedure, which gave 97% purity in a yield of 31% of that expected. 93% of the depleted cells were Thy-1 positive. 7.8×10^6 cells carrying 9100 cpm were injected intravenously to a syngeneic recipient. Total recovery of label when the recipient was killed 21 hours later was 94% of that injected.

D. Discussion

I. Surface Thy-1 on Rat Stem Cells

I have demonstrated here by direct tracing of cell lineages in radiation chimeras that rat stem cells carry Thy-1 at an early stage of development before maturation to T and B lymphocytes. The lymphopoietic assays measure early cells because first, the chimerism is stable (Sect. C II.); second, they precede thymus cells (Sect. C I.) and third, their tissue distribution corresponds with CFU_s [21] and rat CFU_s carry Thy-1 (Sect. C II.). This last observation confirms the findings of

Goldschneider et al. [22, 23] and extends Thierfelder's [24] result showing Thy-1 on the progenitors of rat granulocytes. The rat is not like the mouse, therefore, since clearly Thy-1 does not act as a marker of mature T cells in the rat; it can indeed be found on lymphoid cells of adult thymectomized irradiated bone marrow reconstituted rats [10]. In addition, mouse CFU_s do not bear Thy-1 [25–27]. The discrepancy between the species may possibly be explained by the fact that Thy-1 is heavily glycosylated [11] and the carbohydrate rather than the protein may be the functional part of the molecule. Alternatively the molecule may just be without function at this stage. Whichever way, it may prove to be a useful handle to manipulate stem cell populations experimentally.

II. Thy-1 in the Periphery and the Implications of Double-marked Cells

Thy-1 is present on only a small proportion of rat lymphocytes in the periphery, judged by staining with saturating amounts of fluorescent anti-Thy-1 [9, 28]. It is hard to give a precise figure because the distribution of fluorescence intensity analyzed on the cell sorter is smeared and is less bright than the modal intensity of bone marrow cells, making it difficult to select an appropriate cut-off. It lies somewhere between 5 and 15 percent of all thoracic duct lymphocytes. Both B and T surface markers may be found on these Thy-1 positive cells. The relatively weak, smeared profile implies a heterogeneous population carrying a low density of Thy-1 and taken together with the small frequency of stained cells strongly suggests that Thy-1 is an antigen on the decline in the periphery. Exactly the converse holds for surface immunoglobulin where it is the marrow that has the weak, heterogeneous monotonic labeling [10] and the peripheral (thoracic duct) cells that show strong bimodal labeling. Those cells in marrow and spleen [10] and the thoracic duct [9] that carry both markers are most likely to represent an intermediate stage, though the sequence

$$\text{Thy-1}^{+}\text{sIg}^{-} \longrightarrow \text{Thy-1}^{+}\text{sIg}^{+} \longrightarrow \text{Thy-1}^{-}\text{sIg}^{+}$$

remains to be formally demonstrated. Ia is evidently also a differentiation antigen, not being present on B stem cells (Sect. C III.) nor (in the mouse) on CFU_s [29] but appearing on peripheral B cells. It may be anticipated that a similar scheme to the sequence outlined above for immunoglobulin may also hold for Ia, though perhaps at a slightly later stage since in ontogeny it is delayed [30, 31].

As for the T cell alloantigen Pta there are essentially no cells in marrow or thymus that display it [6, 7, 9]. The 0.5% of marrow cells that are Pta-positive could easily be "contaminating" mature cells from the recirculating pool [32]. Though the absence of Pta from T lymphoid stem cells has not been formally tested, the fact that less than 1% of thymus cells stain for Pta makes it very likely that it too is a differentiation antigen. It might therefore be expected that an intermediate population of Thy-1^{+} Pta^{+} should exist, but studies aimed at identifying such doubly-marked cells in the periphery have so far proved inconclusive largely because of the heterogeneity of Thy-1 fluorescence intensity referred to above. This makes it difficult to be certain what is the proper criterion to define a peripheral cell as Thy-1 positive.

III. Conclusion

The findings in these experiments may be summarized diagrammatically as in Fig. 2. The lymphopoietic assays provide the evidence for the overall transition from early stem cells, carrying Thy-1, through to mature T and B cells in the periphery. The rest of the scheme rests on deductions from the tissue distribution of cells carrying the differentiation antigens under study in this paper and from such doubly-marked cell populations as have been revealed so far. Since CFU_s [22] and granulocyte precursors [24] also carry Thy-1, branches to other hemopoietic lines could justifiably be included at an early stage on the scheme. Additionally mast cells, at least those in bone marrow, bear Thy-1 [10] warranting a branch to them also.

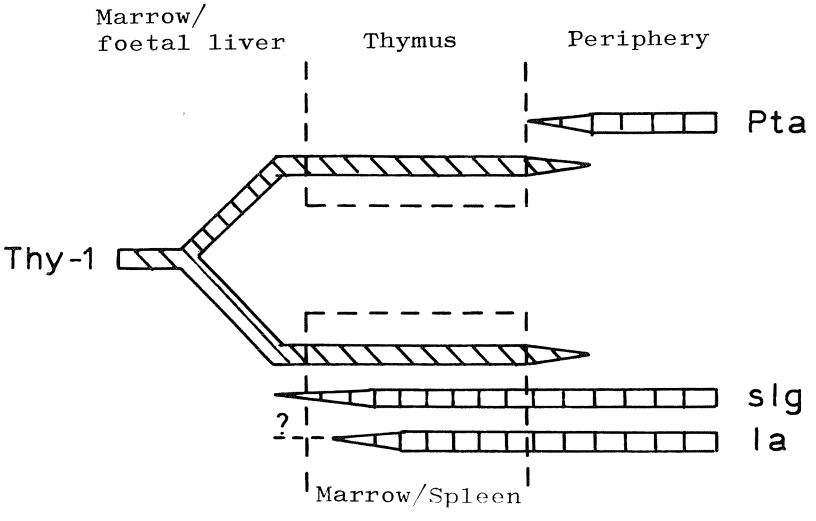


Fig. 2. Summary of surface marker changes during rat lymphocyte development

It should be stressed that most rat marrow Thy-1 positive cells must be somewhere between the pluripotential stage, which is unlikely to be present at a frequency greater than 1% or so of nucleated cells [33], and the mature B lymphocyte that has acquired its surface immunoglobulin. Having subtracted these about three quarters of the whole Thy-1 positive population remains. The migration studies (Sect. C.IV) suggest that these are already determined not to go to the thymus. The fate remains to be discovered of this substantial group of cells, which in the whole juvenile rat (4–5 weeks of age) amounts very approximately to 7 to 10×10^8 cells if the femur is representative of all marrow [9]. They are likely to be the cells in which commitment to the B lineage occurs.

Acknowledgements:

I am very grateful to Marjorie Fowler for her patient technical help, to Alan Williams and Don Mason for discussions and unstinting supplies of antibodies and to Geoff Butcher for PVG-Pta_{DA} rats and antisera and access to his unpublished data.

References

1. Boyse, E. A., Old, L. J.: Some aspects of normal and abnormal cell surface genetics. Annu. Rev. Genet. *3,* 269–290 (1969)
2. Williams, A. F.: Differentiation antigens of the lymphocyte cell surface. Contemp. Top. Mol. Immunol. *6,* 83–114 (1977)
3. Williams, A. F., Galfré, G., Milstein, C.: Analysis of cell surfaces by xenogeneic myeloma-hybrid antibodies: differentiation antigens of rat lymphocytes. Cell *12,* 663–673 (1977)
4. Micklem, H. S., Ford, C. E., Evans, E. P., Gray, J.: Interrelationships of myeloid and lymphoid cells: studies with chromosome marked cells transferred into lethally irradiated mice. Proc. R. Soc. Lond. B *165,* 78–102 (1966)
5. Nezlin, R., Rokhlin, O.: Allotypes of light chains of rat immunoglobulins and their application to the study of antibody biosynthesis. Contemp. Top. Mol. Immunol. *5,* 161–184 (1976)
6. Howard, J. C., Scott, D. W.: The identification of sera distinguishing marrow-derived and thymus-derived lymphocytes in the rat thoracic duct. Immunology *27,* 903–922 (1974)
7. Butcher, G. W.: Ph. D. Thesis, University of Cambridge 1979
8. Hood, L., Gray, W. R., Sanders, B. G., Dreyer, W. J.: Light chain evolution. Cold Spring Harbor Symp. Quant. Biol. *32,* 133–146 (1967)
9. Hunt, S. V.: unpublished data
10. Hunt, S. V., Mason, D. W., Williams, A. F.: In rat bone marrow Thy-1 antigen is present on cells with membrane immunoglobulin and on precursors of peripheral B lymphocytes. Eur. J. Immunol. *7,* 817–822 (1977)
11. Williams, A. F., Barclay, A. N., Letarte-Muirhead, M., Morris, R. J.: Rat Thy-1 antigens from thymus and brain: their tissue distribution, purification and chemical composition. Cold Spring Harbor Symp. Quant. Biol. *41,* 51–61 (1977)
12. Barclay, A. N., Letarte-Muirhead, M., Williams, A. F.: Purification of the Thy-1 molecule from rat brain. Biochem. J. *151,* 699–706 (1975)
13. Morris, R. J., Letarte-Muirhead, M., Williams, A. F.: Analysis in deoxycholate of three antigenic specificities associated with the rat Thy-1 molecule. Eur. J. Immunol. *5,* 282–285 (1975)
14. Letarte-Muirhead, M., Barclay, A. N., Williams, A. F.: Purification of the Thy-1 molecule, a major cell surface glycoprotein of rat thymocytes. Biochem. J. *151,* 685–697 (1975)
15. McMaster, W. R., Williams, A. F.: Identification of Ia glycoproteins in rat thymus and purification from rat spleen. Eur. J. Immunol. *9,* 426–433 (1979)
16. Abramson, S., Miller, R. G., Phillips, R. A.: The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. J. Exp. Med. *145,* 1567–1579 (1977)
17. Williams, A. F.: Many cells in rat bone marrow have cell surface Thy-1 antigen. Eur. J. Immunol. *6,* 526–528 (1976)
18. Hunt, S. V., Williams, A. F.: The origin of cell surface immunoglobulin of marrow-derived and thymus-derived lymphocytes of the rat. J. Exp. Med. *139,* 479–496 (1974)
19. Owen, J. J. T., Raff, M. C., Cooper, M. D.: Studies on the generation of B lymphocytes in the mouse embryo. Eur. J. Immunol. *5,* 468–473 (1975)
20. Yoshida, D., Osmond, D. G.: Homing of bone marrow cells. Localisation and fate of newly formed cells in lymphocyte-rich marrow fractions injected into lethally x-irradiated recipients. Transplantation *25,* 246–251 (1978)
21. Metcalf, D., Moore, M. A. S.: Haemopoietic cells. In: Frontiers of biology. Neuberger, A., Tatum, E. L. (eds.), p. 71. Amsterdam: North-Holland 1971
22. Goldschneider, I., Gordon, L. K., Morris, R. J.: Demonstration of Thy-1 antigen on pluripotent hemopoietic stem cells in the rat. J. Exp. Med. *148,* 1351–1366 (1978)
23. Goldschneider, I.: Heterologous antiserum to rat lymphohemopoietic precursor cells. J. Immunol. *118,* 2040–2046 (1977)
24. Thierfelder, S.: Haemopoietic stem cells of rats but not of mice express Thy-1.1 alloantigen. Nature *269,* 691–693 (1977)
25. Tyan, M. L., Ness, D. B.: Modification of the mixed leukocyte reaction with various antisera. Transplantation *13,* 198–201 (1972)
26. Tyan, M. L.: Modification of severe graft-versus-host disease with antisera to the Θ antigen or to whole serum. Transplantation *15,* 601–604 (1973)

27. Cerny, J.: Stimulation of bone marrow haemopoietic stem cells by a factor from activated T cells. Nature *249*, 63–65 (1974)
28. Acton, R. T., Morris, R. J., Williams, A. F.: Estimation of the amount and tissue distribution of rat Thy-1.1 antigen. Eur. J. Immunol. *4*, 598–602 (1974)
29. Basch, R. S., Janossy, G., Greaves, M. F.: Murine pluripotential stem cells lack Ia antigen. Nature *270*, 520–522 (1977)
30. Duvall, E.: Studies on the development of immunocompetent cells. D. Phil. thesis, University of Oxford 1976
31. Hämmerling, U., Chin, A. F., Abbott, J.: Ontogeny of murine B lymphocytes: sequence of B-cell differentiation from surface-immunoglobulin negative precursors to plasma cells. Proc. Natl. Acad. Sci. USA *73*, 2008–2012 (1976)
32. Howard, J. C., Scott, D. W.: The role of recirculating lymphocytes in the immunological competence of rat bone marrow cells. Cell. Immunol. *3*, 421–429 (1972)
33. Dunn, C. D. R.: The differentiation of haemopoietic stem cells. Ser. Haematologica *4*, 12 (1971).

Discussion

Simonsen: Could you reconstitute the irradiated animals with marrow treated with anti-Thy-1 plus complement? Does it leave any stem cells at all?
Hunt: I am suspicious of using complement-mediated cytotoxicity as a way of determining whether an antigen is present on a cell or not. It is going about it the wrong way if you delete the cell you are interested in and find that you get no reconstitution. The advantage of the sorter is that you can obtain the cell you think is the precursor and see what its activity might be. The other reason for avoiding complement-mediated cytotoxic activity is that we have no idea of the requirement of antigen density in order to kill cells. We have treated marrow with monoclonal anti-Thy-1 antibody and shown that it knocks out its CFUs.
Riethmüller: What information do you have on the physical characterisation of Thy-1? It is also on myeloid cells, on brain cell and on certain fibroblasts. There seems to be some general importance to the molecule.
Hunt: I don't know in which tissues Thy-1 is really playing a functional role. Thy-1 has been purified by Alan Williams and his colleagues. They have isolated it from thymus and from brain [11–14]. It has a molecular weight of approximately 18,000, of which about two thirds is protein and one third is carbohydrate. There are probably 105 amino acids in Thy-1 and it is the protein that contains the antigenic determinants. The carbohydrate comprises 3 oligosaccharide chains, two of which are asparagine-linked and one of which is serine-linked. There are about 28 sugar residues which means that the molecule is very heavily glycosylated.

Bone Marrow Transplantation into Recipients Sensitized Against Donor-type T Cells

S. Thierfelder, E. Thiel, G. Hoffmann-Fezer and H. Rodt

A. Summary

Immunization of prospective bone marrow recipients with thymocytes of the marrow donor strain prevented subsequent hemopoietic engraftment of the marrow. Immunization with thymocytes of a third party strain sharing the marrow donor's theta alloantigen but not his histocompatibility antigens permitted chimaerism and suppressed GVH in mice.

Bone marrow of rat strains of the Th-1.1 group did not take in mice sensitized against mouse Th-1.1 thymocytes. This observation lead to the conclusion that hemopoietic stem cells of rats but not of mice express an early thymic and even prethymic antigen. This antigen is not restricted to the Th-1.1 specificity: rabbit anti-rat thymocyte globulin absorbed in a way which removed stem cell toxicity from a rabbit anti-mouse thymocyte globulin prevented hemopoietic engraftment when incubated with rat bone marrow before transfer to irradiated mice. Serologically this antiserum reacted with thymocytes as well as lymphocytes in the rat bone marrow but less with lymphnode cells. An antiserum against rat lymphnode cells was rendered T cell specific by absorption. It defined a postthymic T antigen lacking on stem cells, bone marrow lymphocytes and thymus. It labeled lymphocytes in the interfollicular T dependent area of lymphnodes by an immunohistochemical method. T cells in rats were thus found to express a prethymic and/or a postthymic antigen.

Implications of theta alloantigens for the suppression of GVH in polytransfused bone marrow grafted patients are discussed.

B. Introduction

Acute graft-versus-host reactions (GVH) are known to be caused by T antigen bearing lymphocytes in the donor's marrow. Suppression of GVH can therefore be achieved by pretreating the donor's marrow with specifically absorbed heterologous anti-T cell globulin [1, 2]. Since this approach did so far not entirely eliminate chronic mortality in the most incompatible situation, e.g. C57Bl/6 to CBA in mice, or – as shown in this volume in a certain percentage of DLA-incompatible dogs – the question was raised whether a single incubation of the donor marrow with anti-T could be replaced by a more prolonged treatment of T cells in the marrow graft.

The following investigations concern the possibility of a presensitization of the prospective bone marrow recipient against T cells in the donor marrow.

Immunization of marrow recipient mice with T lymphocytes of the marrow donor strain induces H-2 antibodies directed against H-2 tissue antigens on T lymphocytes but also on hemopoietic stem cells. This would of course prevent hemopoietic engraftment.

An approach employing third party sensitization is therefore presented [3] which circumvents this complication and leads to marrow engraftment with little or no GVH. Our studies also reveal that rats in contrast to mice express two T antigens, a prethymic T antigen, which occurs on stem cells and a postthymic antigen which occurs on nonthymic T cells but not on hemopoietic stem cells of rats.

C. Materials and Methods

Adult Th-1.1 mouse strains AKR/J, CBA-T66 and (C57Bl/6 × CBA-T6)F_1 were obtained from The Jackson Laboratory, Bar Harbor, Maine and Th-1.2 AKR/Cum mice from Cumberland View Farms, Clinton, Tennessee. The two AKR sublines are antigenically similar with respect to markers Ly-1, Ly-2, Ly-3, TL, H-2, G_{IX} and Gross cell surface antigen while differing for Th-1 [4]. A ^{AKR}(Th-1.1) mice were produced from mice originally supplied by Dr. E. A. Boyse of the Sloan-Kettering Institute, New York. They are congenic to A/J and differ only at the Th-1 locus.

Inbred Lewis rats were produced in our laboratory: 90% of their thymocytes were lysed by anti-Th-1.1 but not by anti-Th-1.2 in the microcytotoxicity test. All rat strains tested sofar are Th-1.1 [5, 6].

Irradiation. Mice were exposed in Lucite containers to 800R using ^{137}Cs (Wälisch-Miller, HWM-D-2000; 131 R/min, opposing sources, each target distance 35 cm).

Presensitization of the prospective marrow recipients consisted of three i.p. injections of 10^8 rat thymocytes or thymocytes of AKR mice given 5 days apart.

Transplantation of cells. 20×10^6 mouse bone marrow together with 50×10^6 spleen cells or 10^8 rat bone marrow cells were obtained by flushing femurs and tibias of the donors with minimal essential medium or by mincing spleens through a grid. The cells were transferred 24 hr after irradiation.

Analysis of chimaerism. Cells were followed up in the peripheral blood of the recipient animals by selective alkaline phosphatase staining of rat granulocytes [7] and in selected cases by chromosomal analysis of the T-6 marker chromosome [8]. Blood smears were done at least twice between day 10 and 20 after transplantation of rat marrow.

Homologous and heterologous antisera against T cells. CBA (Th-1.2) mice were injected i.p. weekly with 10^8 thymocytes of AKR/J (Th-1.1) or AKR/Cum (Th-1.2) strain for 3–4 weeks after which a cytotoxic titer of over 1:128 was noted. The serum was inactivated at 56C for 45 min.

Rabbits were immunized with 10^8 thymocytes or lymphnode cells on day 1, 14, 21, 23, 24. The serum was absorbed as indicated in results and fractionated [2] and used at a concentration of 10 mg/ml. The heterologous antisera had a titer of over 1:64 in the quantitative complement fixation test. All sera were used for incubation of rat bone marrow without addition of complement.

Immunohistochemistry. T antigen in thymus and lymphoid tissue was demonstrated on frozen sections using the unlabeled enzyme peroxidase-anti-peroxidase complex method (PAP) [10] which was modified for surface marker histology [9].

D. Results

I. Allogeneic Bone Marrow Transplantation into Presensitized Mice

Experimental approach.

Prospective bone marrow recipients were sensitized against thymocytes from a third party strain (mouse or rat) sharing the theta alloantigen of the bone

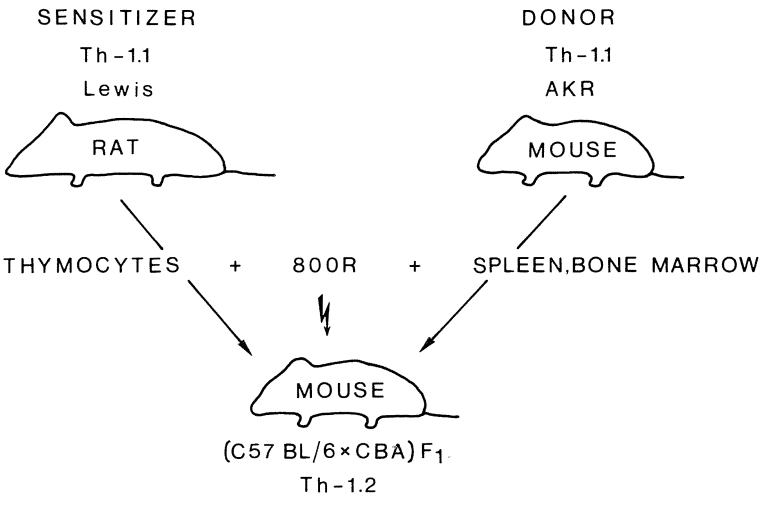


Fig. 1. Experimental design of xenogeneic presensitization of Th-1.2 marrow recipients against Th-1.1 donor type alloantigen

marrow donor strain but not his histocompatibility antigens. They were then irradiated with 800 R and transfused with 20 million bone marrow together with 50 million spleen cells (Fig. 1). Under these conditions bone marrow transplantation into H-2 incompatible Th-1.2 or Th-1.1 was tested.

1. Chimerism (Table 1)

Immunization of $(C57Bl/6 \times CBA\text{-}T_6)F_1$ mice (Th-1.2) with thymocytes of the bone marrow donor AKR/J Th-1.1 strain prevented hemopoietic engraftment. No chimerism was found (Table 1). Most of the irradiated marrow recipients were dead 2 weeks after transplantation.

Donor cell mitoses could be demonstrated if the same strain had been preimmunized with Lewis rat thymocytes (Th-1.1) before transplantation of marrow from AKR (Th-1.1) mice.

T cell chimerism using anti-Th.1 antisera for typing could also be demonstrated 2 weeks after transplantation when $(A^{AKR} \times AKR/J)F_1$ mice (Th-1.1) were immunized with thymocytes of C57Bl/6 mice before transfusion of marrow from A (Th-1.2) mice.

Table 1. Chimerism after bone marrow transplantation between Th-1 incompatible mice presensitized against donor-type alloantigen

Recipient		Immunization		Donor		Take chimerism
Th-1.1	Th-1.2	Th-1.1	Th-1.2	Th-1.1	Th-1.2	
	$(C57BL/6 \times CBA\text{-}T6)F_1$	AKR/J		AKR/J		−
	$(C57BL/6 \times CBA\text{-}T6)F_1$	Lewis		AKR/J		+
	CBA-T66	AKR/J		A^{AKR}		+
$(A^{AKR} \times AKR/J)F_1$			C57BL/6		A	+

Chimerism was also found if CBA-T_{66} (Th-1.2) preimmunized with AKR/J thymocytes (Th-1.1) was transfused with A^{AKR} (Th-1.1) (Table 1).

2. Graft-Versus-Host Mortality

(C57BL/6×CBA)F_1 mice transfused with spleen and bone marrow of AKR mice died of acute GVH within 2 weeks after transplantation. Presensitization against Lewis rat thymocytes protected 75% from acute and chronic mortality (Fig. 2). 100 days after transplantation the recipient mice were grafted with skin of AKR mice (not shown) which was not rejected on day 200 post transplantation at which time 6 of 6 (C57BL/6×CBA-T6) mice were full chimeras (> 95%) according to chromosomal analysis.

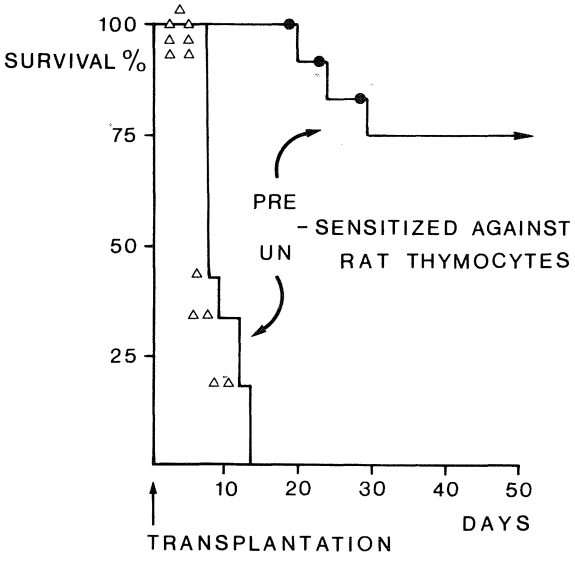


Fig. 2. Survival after transplantation of AKR spleen marrow into (C57BL/6×CBA)F_1 mice presensitized or not against rat thymocytes

A dose response between the degree of protection from GVH and the times of immunization with rat thymocytes was noted (Fig. 3). One injection of rat thymocytes conferred little protection when compared with the unsensitized control. After 2 or 3 booster injections a definitely better survival was noted.

Bone marrow transplantation in the reverse combination (Th-1.2 into Th-1.1) lead to 100% mortality. Sensitization against a third party strain sharing the marrow donor's Th-1 alloantigen conferred an almost complete protection from GVH mortality (Fig. 4).

In order to exclude an unspecific protective effect from the preimmunization procedure itself prospective marrow recipients were presensitized against thymocytes of congeneic AKR strains sharing either the donor's or the recipient's Th-1 alloantigen (Fig. 5). While CBA (Th-1.2) recipients of A^{AKR} (Th-1.1) spleen and bone marrow presensitized against AKR/Cum (Th-1.2) thymocytes died of acute GVH within 2 weeks after transplantation, preimmunization against donor type theta alloantigen expressing AKR/J (Th-1.1) thymocytes protected over 80% of the recipients from GVH mortality (Fig. 6).

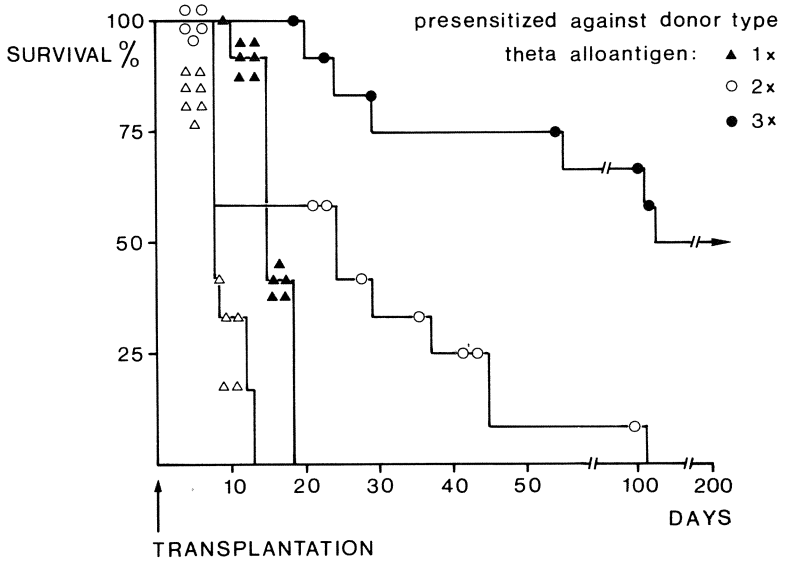


Fig. 3. Survival after transplantation of spleen marrow into H-2 incompatible mice. Recipient mice were unsensitized (△) or presensitized once (▲), twice (◯) or 3 times (●) against donor type thetaalloantigen

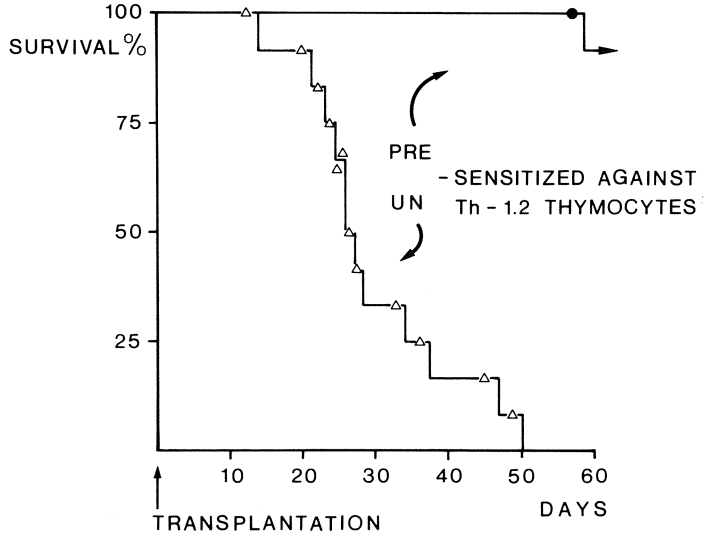


Fig. 4. Survival after transplantation of A^{AKR} (Th-1.2) spleen marrow into $(A^{AKR} \times AKR/J)F_1$ (Th-1.1) mice presensitized or not against C57BL/6 (Th-1.2) thymocytes

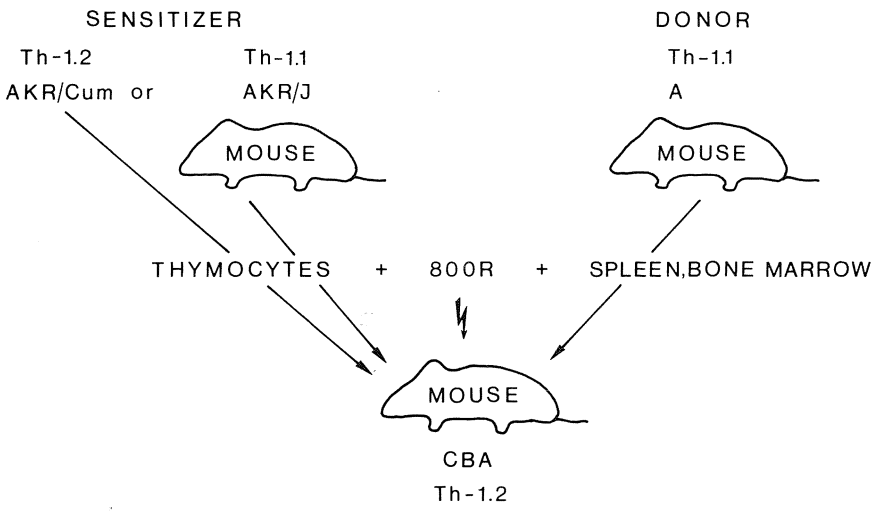


Fig. 5. Experimental design of allogeneic presensitization of Th-1.2 marrow recipients against Th-1.2 or Th-1.1 thymocytes

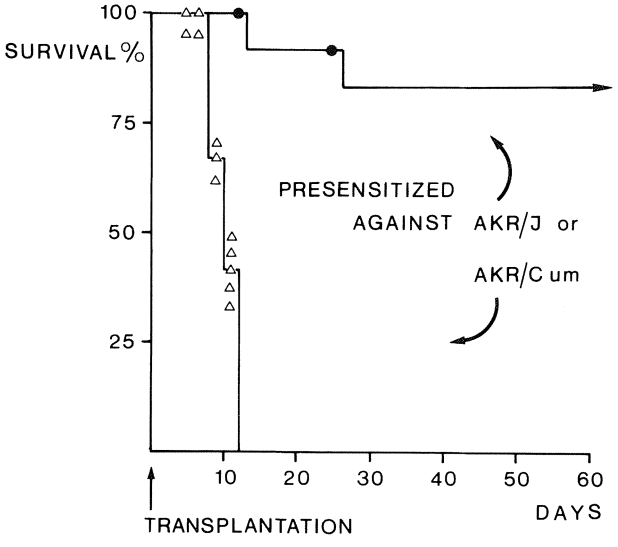


Fig. 6. Survival after transplantation of A (Th-1.1) spleen marrow into CBA (Th-1.2) mice presensitized against AKR/J (Th-1.1) or AKR/Cum (Th-1.2) thymocytes

II. Xenogeneic Bone Marrow Transplantation into Presensitized Mice

Experimental approach (Fig. 7).

Prospective bone marrow recipient mice were sensitized against thymocytes from a third party mouse strain sharing the Th-1.1 alloantigen of the rat bone

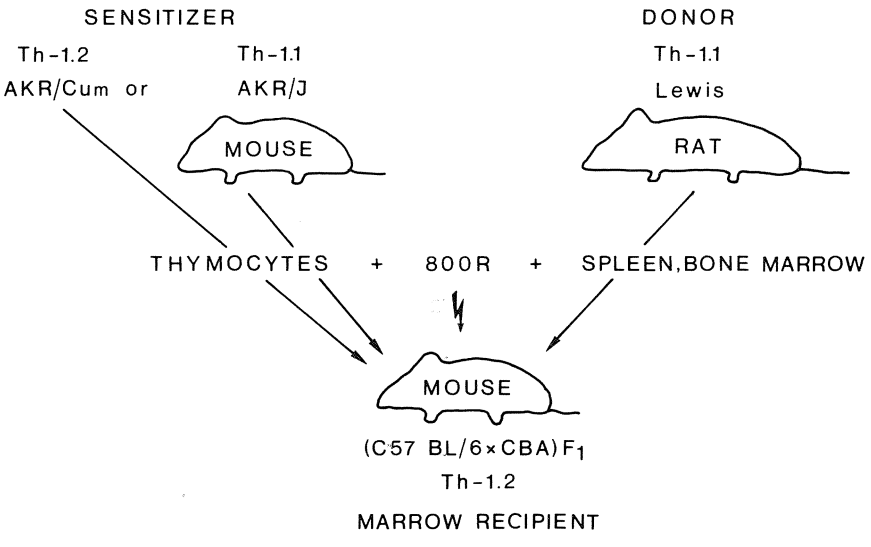


Fig. 7. Experimental design of presensitization of Th-1.2 mice against murine Th-1.2 or Th-1.1 thymocytes before rat (Th-1.1) bone marrow transplantation

marrow donor strain. They were then irradiated and transfused with 10^8 bone marrow of Lewis rats.

1. Chimerism (Table 2)

Immunization of (C57BL/6×CBA)F_1 mice (Th-1.2) with thymocytes of AKR/J (Th-1.1) prevented a take of subsequently transfused Lewis rat bone marrow. The majority of mice died with a histologically empty bone marrow and spleen and no alkaline positive rat granulocytes. In contrast unsensitized mice or mice immunized with AKR/Cum (Th-1.2) were chimeric with rat mitoses and alkaline phosphatase staining rat granulocytes. In order to exclude a presensitization of the bone marrow recipients against tissue antigens other than Th-1.1 AKR/Cum (Th-1.2) were immunized with thymocytes of congeneic AKR/J (Th-1.1) which again prevented hemopoietic engraftment of transfused rat marrow.

2. Pretreatment of Rat Marrow with Anti-T (Table 3)

10^8 Lewis rat bone marrow cells were incubated with 0.25 ml anti-Th-1.1 or anti-Th-1.2 at room temperature for 30 min. While pretreatment with anti-Th-1.2 did not affect rat bone marrow transplantation, incubation with anti-Th-1.1 prevented hemopoietic engraftment of rat marrow in mice.

Table 2. Chimerism after transfusion of rat bone marrow into irradiated Th-1.2 mice preimmunized with Th-1.2 or Th-1.1 mouse thymocytes or B-cell incompatible spleen cells

Recipient Th-1.2	Immunization		Donor	Take chimerism
	Th-1.1	Th-1.2	Th-1.1	
(C57BL/6×CBA)F_1	AKR/J		Lewis	−
(C57BL/6×CBA)F_1		AKR/Cum	Lewis	+
AKR/Cum	AKR/J		Lewis	−

Table 3. Chimerism in irradiated Th-1.2 mice after transfusion of rat marrow preincubated with homologous anti-theta or heterologous anti-T

Recipient Th-1.2	Incubation with antiserum	Donor Th-1.1	Take chimerism
(C57 BL/6×CBA)F_1	CBA anti-AKR/J (Th-1.1)	Lewis	−
(C57 BL/6×CBA)F_1	CBA anti-AKR/Cum (Th-1.2)	Lewis	+
(C57 BL/6×CBA)F_1	Rabbit anti-rat-T (thymus-T)	Lewis	−
(C57 BL/6×CBA)F_1	Rabbit anti-rat-T (peripheral T)	Lewis	+

If rat marrow was pretreated with heterologous rabbit anti-rat-thymocyte globulin absorbed with liver and 5 times with spleen of AKR mice no chimerism was observed following transfusion into (C57BL/6×CBA)F_1 mice, whereas incubation of rat marrow with rabbit anti-rat lymphnode serum (anti-peripheral T serum) absorbed 5 times with rat thymocytes did not prevent subsequent xenogeneic chimerism.

3. Specificity of Absorbed Rabbit Anti-Rat T Cell Globulin

Rabbits were immunized with rat T cells of either thymus or lymphnode (s. Materials and Methods). The anti-lymphnode serum was absorbed with liver homogenate and 5 times with thymus. The anti-thymocyte serum was absorbed with liver and 5 times with spleen. The globulin fraction of these antisera were tested in quantitative complement fixation test against rat bone marrow, thymus and lymphnode cells.

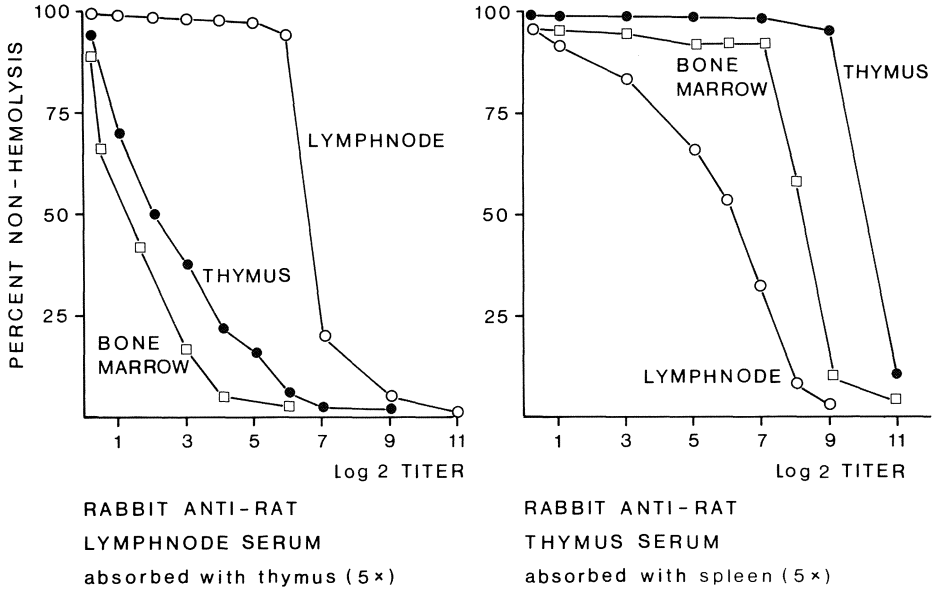


Fig. 8. Complement fixing activity of rabbit anti-rat lymphnode or thymus serum against cell suspensions of lymphnode, thymus or bone marrow

As seen in Fig. 8 the antilymphnode serum was positive with lymphnode cells, showing little cross-reaction with thymus and bone marrow, whereas the anti-thymus serum was more positive with thymus and bone marrow than with lymphnode. These data were compatible with an anti-thymic T cell specificity reacting not only with thymocytes but also with thymic or even prethymic T cells in the rat bone marrow. The anti-lymphnode serum appeared to react with postthymic T cells in the lymphnode at dilutions which no longer showed a specificity for thymocytes.

4. Immunohistochemical Staining of Rabbit Anti-Rat T Cell Globulin

Since the anti-rat lymphnode serum showed little reactivity with thymocytes, its specificity for peripheral T lymphocytes was tested on frozen sections using the unlabeled enzyme method with peroxidase-antiperoxidase complex staining (PAP). The absorbed anti-thymocyte globulin stained T cells in the thymus and the interfollicular thymus dependent area of a lymphnode (Fig. 10a, 11a). At higher dilutions the lymphocytes in the thymus became negative while lymphnode T cells were still clearly labeled with the antiserum (Fig. 10b, 11b). Except for a few migrating T cells the lymphfollicles consisting predominantly of B cells remained unlabeled by both antisera (Fig. 11).

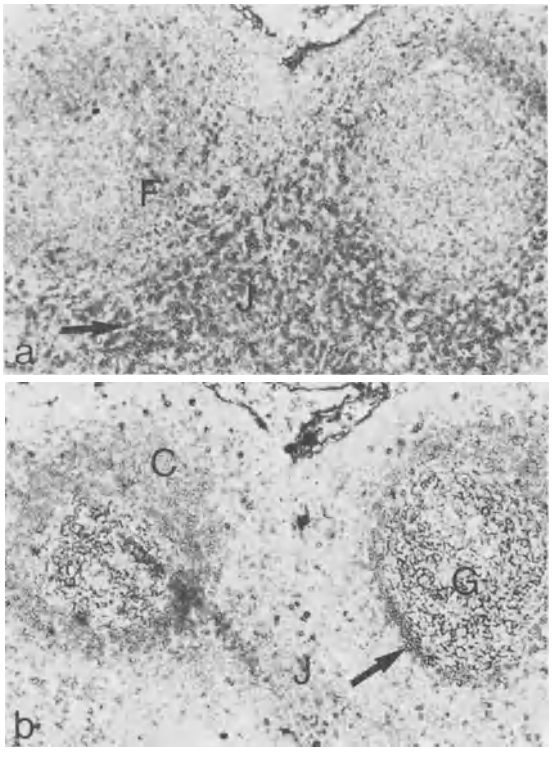


Fig. 9. Example of T and B cell areas. Human palatine tonsil **a** T-lymphocytes (↑) labeled by anti-T in the interfollicular area (J), F = follicle with germinal center, ×105; **b** B-lymphocytes (↑) labeled by anti-IgM in the lymphocytic cap (C) of follicle, J = interfollicular area; G = germinal center with cytoplasmic Ig-staining of dendritic reticulum cells and some B-lymphocytes; ×105

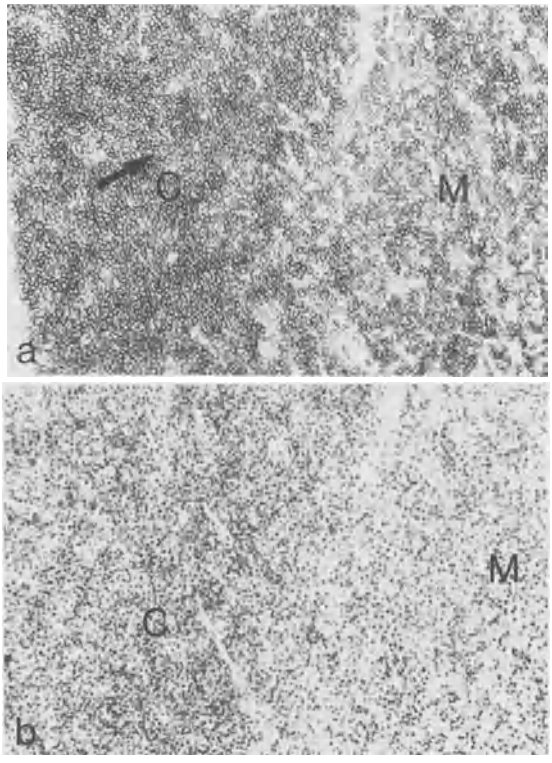


Fig. 10. Rat thymus, C = cortex, M = medulla, **a** most thymocytes labeled by 728 = A-Th (↑), × 165; **b** no labelling of cortical and most medullar thymocytes by 936 b = A-Tp; × 165

E. Discussion

Sensitization of prospective bone marrow recipient mice against T cells in the marrow graft has been found to suppress GVH provided thymocytes of a strain sharing the marrow donor's theta alloantigen but not his histocompatibility antigens were used. We called this suppressive effect 'host-versus-theta graft reaction' [3].

From the resulting chimerism, it can be concluded that hemopoietic stem cells in mice do not express Th-1 alloantigens. Failure of engraftment due to presensitization against antigens on donor-type hemopoietic cells is a very sensitive indicator system. It reveals for instance minor antigen incompatibilities where serological in vitro tests fail [11]. It therefore should also be positive if mouse stem cells express Th-1.1. In contrast to mice it was in fact positive in our transfer studies with rat bone marrow, which did not take in mice presensitized against murine Th-1.1 [12]. The prethymic differentiation of Th-1.1 on rat stem cells is not restricted to the Th-1.1 specificity. Failure of take was likewise observed following treatment of rat marrow with absorbed rabbit anti-rat thymocyte globulin. The antiserum was absorbed in a way which removed all toxicity for mouse stem cells in a rabbit anti-mouse thymocyte globulin. Therefore certain peculiarities of the Th-1.1 antigen, such as its expression on

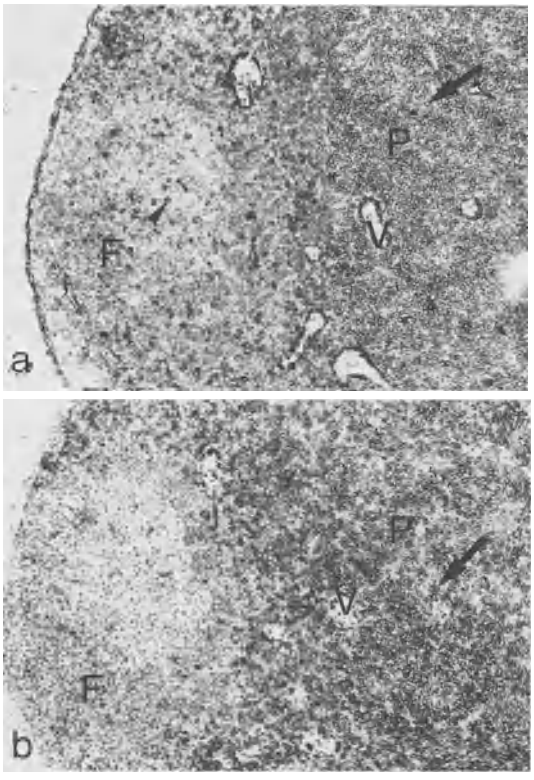


Fig. 11. Rat lymphnode; F = cortical zone with follicle; P = paracortex; V = postcapillary venule, **a** labelling of the majority of paracortical cells (↑) and singular follicle lymphocytes (△) by 728 A-Th, ×130, **b** labelling of many paracortical cells (↑) by 936 b = A-Tp; ×130

a relatively high percentage of bone marrow lymphocytes including early B cells [13], are probably not restricted to Th-1.1 but to the whole T thymocyte antigen structure, as it is defined by the respective heterologous antibody. This T antigen is comparatively little differentiated on 'peripheral' T cells of the rat e.g. spleen and blood lymphocytes. Postthymic T cells differentiate another T antigen which is not present on normal thymocytes [14]. The definition of a T antigen which is lacking on thymocytes was possible by surface marker histology: lymphocytes concentrated in the T dependent interfollicular area of the lymphnode were stained by the respective antiserum. Serologically antiperipheral T serum reacted little with T cells in the bone marrow and spared stem cells, so that hemopoietic engraftment of rat marrow was not prevented by pretreatment with anti-peripheral T.

Suppression of GVH by our host-versus-theta graft approach did not lead to chimaeras deprived of donor type T cells. The anti-T cell effect of the host appeared to subside spontaneously within the first 20 days following transplantation probably because of the replacement of the host's lymphatic system by that of the donor's. 100 days after transplantation the chimaeras rejected third party skin grafts without delay while tolerating skin of the marrow donor strain.

If theta alloantigens exist also in man, host-versus-theta graft effects on GVH can be expected in patients presensitized against T cells in the marrow graft by

blood transfusions from certain donors. In mouse strains Th-1 alloantigens are quite unevenly distributed with Th-1.2 strains prevailing.

If theta alloantigens occur in man and are distributed more evenly than in mice a lower incidence of GVH in multitransfused patients may be expected. In fact, if a lower incidence of GVH in polytransfused aplastic patients successfully grafted with bone marrow could be objectified, theta alloantigen groups as a causative possibility should be considered.

References

1. Rodt, H. V., Thierfelder, S., Eulitz, M.: Suppression of acute secondary disease by heterologous anti-brain serum. Blut 25, 385–389 (1972)
2. Rodt, H., Thierfelder, S., Eulitz, M.: Anti-lymphocytic antibodies and marrow transplantation. III. Effect of heterologous anti-brain antibodies on acute secondary disease in mice. Eur. J. Immunol. *4*, 25–29 (1974)
3. Thierfelder, S., Rodt, H.: Antilymphocytic antibodies and marrow transplantation. V. Suppression of secondary disease by host-versus-Θ-graft-reaction. Transplantation *23*, 87–92 (1977)
4. Acton, R. T., Blankenhorn, E. P., Douglas, T. C., Owen, R. D., Hilgers, J., Hoffmann, H. A., Boyse, E. A.: AKR-mice – genetic variations among sublines. Nature *245*, 8–10 (1973)
5. Douglas, T. C.: Occurrence of a theta-like antigen in rats. J. Exp. Med. *136*, 1054–1062 (1972)
6. Micheel, B., Pasternak, G., Steuden, J.: Demonstration of Θ-AKR differentiation antigen in rat tissue by mouse alloantiserum. Nature *241*, 221–222 (1973)
7. Vos, O., Davids, J. A. G., Weyzen, W. W. H., Bekkum, D. W. van: Evidence for the cellular hypothesis in radiation protection by bone marrow cells. Acta physiol. pharmacol. *4*, 482–486 (1956)
8. Ford, C. E.: The use of marker chromosomes. In: Micklem, H. S., Contit, J. F.: Tissue grafting and radiation, p. 197. New York: Academic Press 1966
9. Hoffmann-Fezer, G., Rodt, H., Eulitz, M., Thierfelder, S.: Immunohistochemical identification of T- and B-lymphocytes delineated by the unlabeled antibody enzyme method. I. Anatomical distribution of Θ-positive and Ig-positive cells in lymphoid organs of mice. J. Immunol. Methods *13*, 261–270 (1976)
10. Sternberger L. A., Cuculis: Method for enzymatic intensification of the immunocytochemical reaction without use of labelled antibodies. J. Histochem. Cytochem. *17*, 190 (1969)
11. Storb, R., Epstein, R. B., Rudolph, R. H., Thomas, E. D.: The effect of prior transfusion on marrow grafts between histocompatible canine siblings. J. Immunol. *105*, 627–633 (1970)
12. Thierfelder, S.: Haemopoietic stem cells of rats but not of mice express Th-1.1 alloantigen. Nature *269*, 691–693 (1977)
13. Hunt, S. V., Mason, D. W., Williams, A. F.: Rat bone marrow Thy-1 antigen is present on cells with membrane immunoglobulin and on precursors of peripheral B-lymphocytes. Eur. J. Immunol. *7*, *11*, 817–823 (1977)
14. Goldschneider, I.: Antigenic relationship between medullary thymocytes and a subpopulation of peripheral T cells in the rat: Description of a masked antigen. Cell. Immunol. *16*, 269–284 (1975)

Discussion

Riethmüller: Did the transplanted mice exhibit spontaneous cytotoxic activity?
Thierfelder: This was not tested.
Storb: We had looked several years ago as to whether the pretransfused aplastic patients with sustained engraftment had a lower incidence of graft-versus-host disease and a better survival than the

non-transfused patients. The indication at that time was no. Now we have more patients and I looked infact just last week at the first 156 aplastic patients transplanted, among whom there are 30 untransfused patients and it came out that the transfused patients had a worse survival.

Thierfelder: Of course the pretransfused patients as such – this is comprehensible – have a poorer prognosis. The only question is, not a postulate, or to turn it the other way round, if a lower incidence of GVH can be objectified in the polytransfused patients this could be explained by theta alloantigen groups.

Waldman: In the mice which had been immunized against the alloantigens of T cells do they develop immunocompetent T cells eventually?

Thierfelder: Yes, they reject third-party skin grafts

Kindred: Do you know whether the T cells which have developed in these mice, are of host or donor type?

Thierfelder: The T cells were of donor type, so the thymus of the Th-1.2 recipient conferred immunocompetence and induced differentiation into Th-1.1 T cells.

Knapp: Do the sensitized mice have antibodies and of what specificity?

Thierfelder: The specificity of the antibodies is depending on the model – Th-1.1 or Th-1.2, but the antibodies themselves do not persist long after bone marrow transplantation because the recipients are irradiated and finally the lymphoid tissue of the recipient is being substituted by that of the donor. Thus the antibody production subsides spontaneously.

Prevention of Graft Versus Host Reactions and Conditioning of Recipients for Bone Marrow Transplantation in Chickens

J. Ivanyi

A. Introduction

Some of the earliest work on graft-versus-host reactions (GVHR) was accomplished in the chick embryo (Simonsen, 1957). The high sensitivity of chick embryonal splenomegaly and chorioallantoic membrane (CAM) pock reactions enabled the demonstration of GVHR with as few as 50 lymphocytes (Nisbet et al., 1969). This observation focused attention on the high frequency of GVHR reactive cells, and was later observed in mammalian species. The contribution of host-derived proliferation to the GVHR in splenomegaly reactions declines from 90 to 10% between days 16–19 of incubation (Owen et al., 1965) whereas in CAM pock reactions remains constantly above 90% (Weber, 1970). Both GVHR (Schierman and Nordskog, 1963) and mixed leukocyte reactions (MLR) (Miggiano et al., 1974) are controlled by the B locus which represents the major histocompatibility complex (MHC) of the chicken.

I shall review in the following sections the experiments from this Laboratory which have been reported over the past several years. These experiments demonstrate some of the effects of GVHR on the bursa of Fabricius, describe the prevention of GVHR by pretreatment of allogeneic lymphoid cells with anti-T cell globulin in vitro and analyse B-cell chimeras using Ig-allotype markers.

B. Manifestations of GVHR in Chick Embryos

Allogeneic lymphocytes injected into developing embryonal recipients encounter pluripotential stem cells (Moore and Owen, 1967) and the progeny of lymphoid and hemopoietic cells at various stages of differentiation. The homing of stem cells into various organs follows a closely predetermined sequence, this being day 6–8 for the thymus and day 8–14 for the bursa of Fabricius (Houssaint et al., 1976). Stem cells and descendant lymphoid elements express histocompatibility antigens (Davis et al., 1970) and thus represent the target cells confronting the injected alloreactive T cells. The outcome of this confrontation may be either proliferative or deletive. Proliferative reactions occur in the spleen after intravenous injection or on the CAM after topical application of allogeneic lymphocytes (Simonsen, 1957).

Hypoplasia of the thymus or bursa may be observed during GVHR (Walker et al., 1973; Lydyard and Ivanyi, 1975a) and probably results from an impaired homing of stem cells into the primary lymphoid organs. Experiments have shown that bursal hypoplasia is analogous to the inhibition of murine hemopoietic stem

cells (Blomgren and Andersson, 1972) and both represent a very sensitive quantitative assay of the GVHR.

The intensity of the deletive and proliferative type of reactions following intravenous injection of allogeneic lymphocytes is determined by the age of the embryonal host. Severe bursal hypoplasia with little splenomegaly occurred in 10 day old embryos while less bursal hypoplasia but strong splenomegaly was observed in 14 day old embryos (Fig. 1). Examination of histological preparations of the bursa from embryos undergoing a GVHR showed severe hypoplasia of bursal plicae but normal development of the few residual follicles (Fig. 2a).

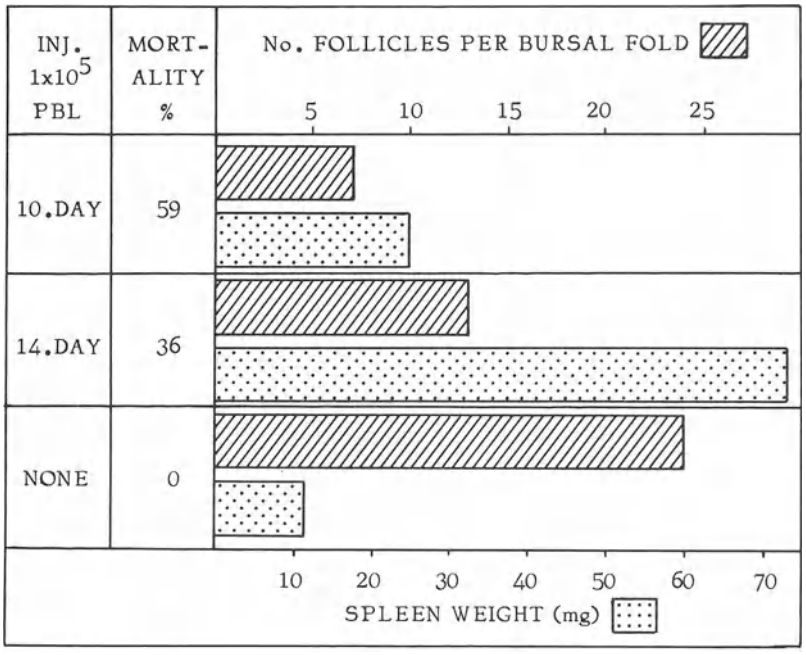


Fig. 1. Manifestations of GVHR in chick embryos. Harvest on 21st day of incubation. Follicles were counted on sections cut across the equatorial plane (Lydyard and Ivanyi, 1975a)

However, bursal plicae from embryos injected at 14–15 days of incubation occasionally contained diffuse infiltrates independent of the follicles (Fig. 1c). Similar proliferative lesions were also described in the thymus and bone marrow (BM) of embryos undergoing GVHR (Walker et al., 1973). These observations suggest that bursal and thymic hypoplasia may result from impaired differentiation of immature lymphoid stem cells in the presence of alloreactive T cells. A similar pathogenesis may play a role in the generation of immunodeficiency in children with demonstrated transplacental passage of maternal lymphocytes (Kadowaki et al., 1966).

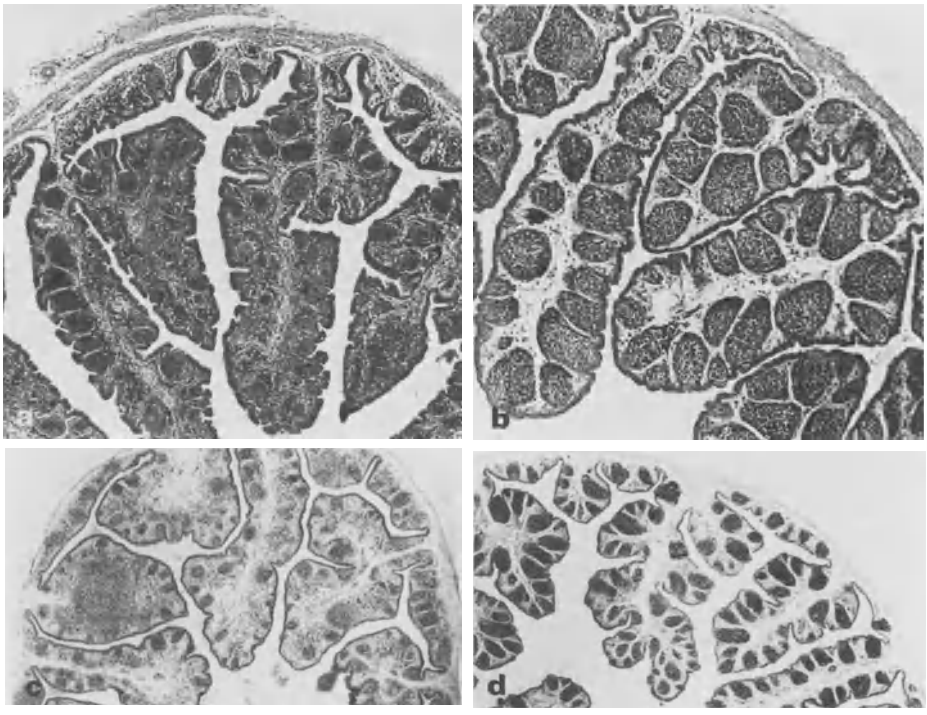


Fig. 2. Hypoplasia of bursal folds in chick embryonal GVHR. Allogeneic lymphocytes injected into 10 day **a** or 14 day **c** old embryos; uninjected controls **b, d.** Sections from bursas harvested 14 days post-hatching **a, b** or from 19 day old embryos **c, d.** (Lydyard and Ivanyi, 1975a)

C. Prevention of GVHR by in Vitro Treatment of Lymphoid Cells with Anti-T Cell or Allogeneic Antisera. The Role of Opsonization for GVHR Suppression

Heterologous antisera against chicken lymphocyte subpopulations have been raised in several laboratories (Forget et al., 1970; McArthur et al., 1971; Lydyard and Ivanyi, 1971; Ivanyi and Lydyard, 1972; Feiglova et al., 1972; Wick et al., 1973; Hudson and Roitt, 1973; Albini and Wick, 1974). Rabbits are immunized effectively with bursal or thymus cells in complete Freund's adjuvant followed by intravenous challenge. Specificity is achieved by cross-absorbing the immune globulins at least twice with 10^9 thymus or bursal cells per 1 ml of serum. The characteristics of specific antibodies prepared in this manner are summarized in Table 1.

It is to be borne in mind that the efficacy and specificity of antisera apparently varies from one test system to another. For example, PHA stimulation is poorly inhibited by anti-T cell globulin (ATG), while GVH-reactive cells are quite sensitive to the same sera (Lydyard and Ivanyi, 1971; Potworowski et al., 1971; Ivanyi and Lydyard, 1972). Since inhibition of GVHR was achieved with

Table 1. Summary of characteristics of rabbit anti-chicken T- and B-cell antibodies (Ivanyi and Lydyard, 1972)

Experimental assay	Treatment in vitro		
	NRG	RATG	RABG
Cytotoxic index[a]: Thymus	0	80	<1
Bursa	0	<1	80
PFC inhibition (%)[b]	<5	<5	90
Relative spleen index[c]	12.5	1.8	NT
% mortality at 2 weeks post hatching	88	8	NT
PHA stimulation index	32	6	NT
Antibody response[d]	6.4	5.6	1.9

[a] Assay with ^{59}Cr-labelled cells
[b] Treatment of anti-SRBC immunized spleen cells
[c] Injection of 2×10^6 BM cells into 13 day old allogeneic embryos
[d] Anti-SRBC $\log_2$ titre of B cell depleted and lymphoid cell reconstituted chickens
NRG = normal rabbit globulin; RATG = rabbit anti-T cell globulin; RABG = rabbit anti-B cell globulin

non-cytotoxic dilutions of rabbit ATG (RATG) and also in the absence of complement, further experiments were performed to ascertain the mechanism of antibody-mediated GVHR-suppression.

Previous studies have suggested that the immunosuppressive action of antilymphocyte sera was due to the opsonizing effect of antibodies. This view was based on the observation that lymphocytes were diverted from the spleen to the liver in the presence of antisera (Taub and Lance, 1968) in correlation with the cytophilic and immunosuppressive potency of the antiserum employed (Greaves et al., 1969) and on the absence of immunosuppression by $F(ab')_2$ or Fab antibody fragments.

Inhibition of chick embryonal GVHR by pretreating the alloreactive lymphocytes by RATG at non-cytotoxic conditions suggested opsonization as a mechanism by which the GVH-reactive lymphocytes were eliminated in vivo. RATG or its $F(ab')_2$ fragments were used for in vitro incubation with allogeneic lymphocytes and the splenomegaly reaction was compared with the spleen (or bursa) → liver lymphocyte diversion in chick embryos (Lydyard and Ivanyi, 1974). The results showed a direct quantitative correlation between the degree of opsonisation as measured by the lymphocyte diversion assay and the embryonal splenomegaly. Furthermore, RATG-$F(ab')_2$ fragments failed to suppress the GVHR and were also ineffective in the diversion assay (Fig. 3). A similar correlation between inhibition of GVHR and opsonizing activity was observed when lymphocytes were treated with anti-B-locus alloantisera (Table 2). Thus we conclude that lymphocyte opsonization mediated by the Fc part of the antibody molecule plays an essential role in the functional elimination of GVH-reactive T lymphocytes.

It has been reported that non-cytotoxic concentrations of ALG have an opsonizing effect resulting in selective homing of lymphocytes into the liver also

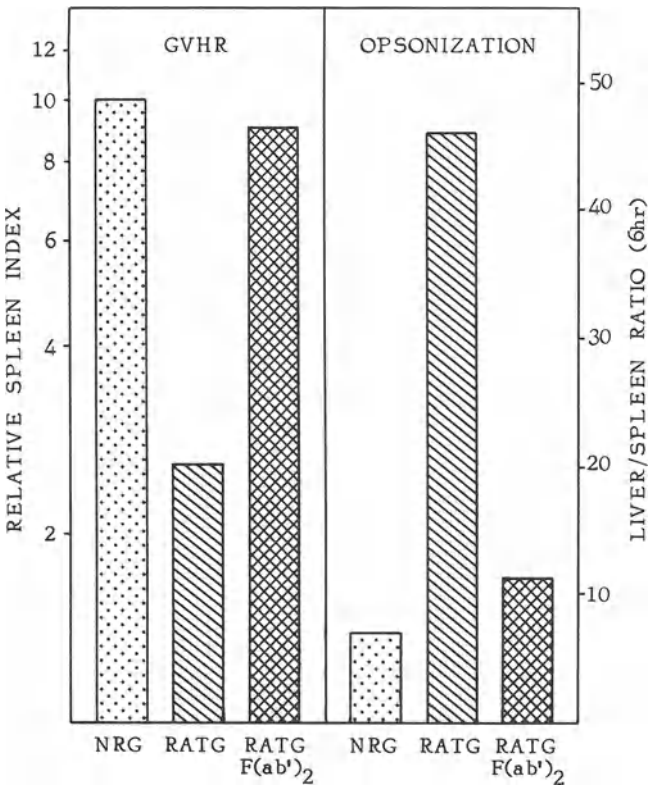


Fig. 3. Correlation between RATG mediated GVHR suppression and lymphocyte opsonization. 10^6 ^{51}Cr labelled lymphocytes after 250 μg/ml globulin treatment in vitro were injected into 13 day old allogeneic embryos. (Lydyard and Ivanyi, 1974)

in man (Chanard et al., 1972). Hence antisera specific against human T lymphocytes (Smith et al., 1973; Touraine et al., 1974; Wick et al., 1976; Brochier et al., 1976; Zola, 1977) and free of anti-stem cell activity (Mosedale et al., 1976) would qualify as adequate reagents for the in vitro preventive treatment of human bone marrow prior to transplantation.

Table 2. Correlation between alloantiserum mediated GVHR suppression and lymphocyte oponisation (Galton, 1978)

Lymphocyte treatment with alloantiserum		% injected radioactivity			Organ ratio		GVHR RSI
		Liver	Spleen	Bursa	L/S	L/B	
CH anti-B14	1/10	81.4	1.6	0.4	50.9	203.5	2.4
	1/100	32.4	5.8	3.2	5.6	10.1	6.6
NCS	1/10	23.2	7.3	4.5	3.2	5.2	10.2

3.5×10^6 ^{51}Cr-labelled B14 PBL incubated with serum at 37° C for 45 minutes was injected i.v. into 13 day old outbred embryos and the organ distribution of radioactivity was determined 24 hours later

D. Prevention of GVHR in Semiallogeneic Radiation Chimeras

Following the conclusion that in vitro treatment with anti-T cell antibody can abrogate mature GVH-reactive cells from bone marrow (BM) cell suspensions, it was necessary to consider the possibility that new GVH-reactive cells could differentiate from BM stem cells subsequent to transplantation. Although prevention of an early acute GVHR episode can be viewed as the first stage of success, determination of the risk and intensity of a delayed reaction required further attention. These aspects were studied in experiments using BM reconstituted lethally irradiated semi-allogeneic recipients (Lydyard and Ivanyi, 1975b).

(CH×B14)F_1 hybrid, 12 day old chickens were repopulated with either RATG or NRG treated BM cells from adult parental B14 strain donors. The GVHR in this model is of the chronic type, manifested by immunosuppression and with demonstrable donor-derived GVH-reactive cells in the peripheral

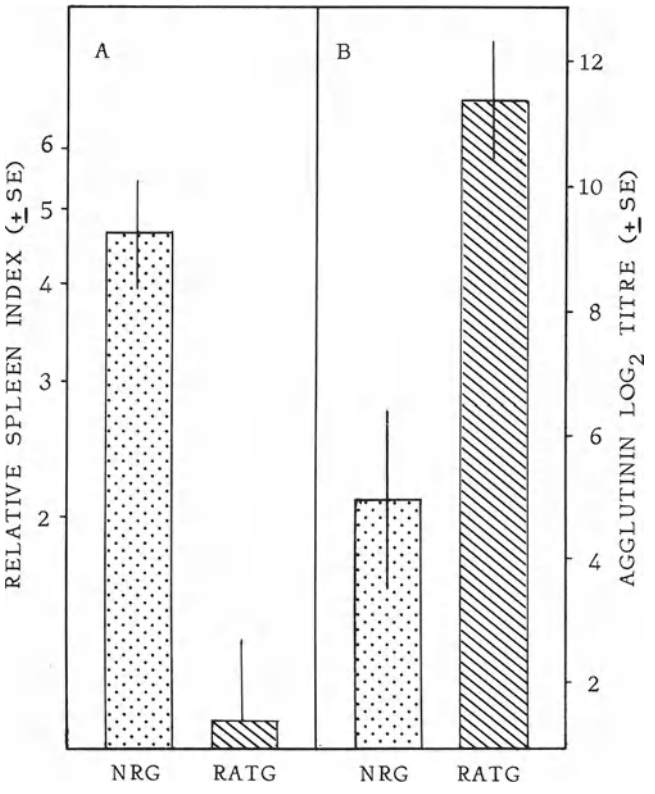


Fig. 4. GVH and antibody responsiveness of parental (B14) bone marrow reconstituted lethally irradiated (CH×B14)F_1 hybrid chimeras. 13 day old 3×500 rads irradiated F_1 hosts were injected with NRG or RATG treated parental BM cells. Six survivors out of 12 injected chickens in each group were tested at the age of 6 weeks for: *(A)* GVHR (donor-derived), induced by 8×10^5 PBL in (CH×B14)F_1 hybrid embryos; *(B)* 7 day antibody response to 4×10^9 SRBC (Lydyard and Ivanyi, 1975b)

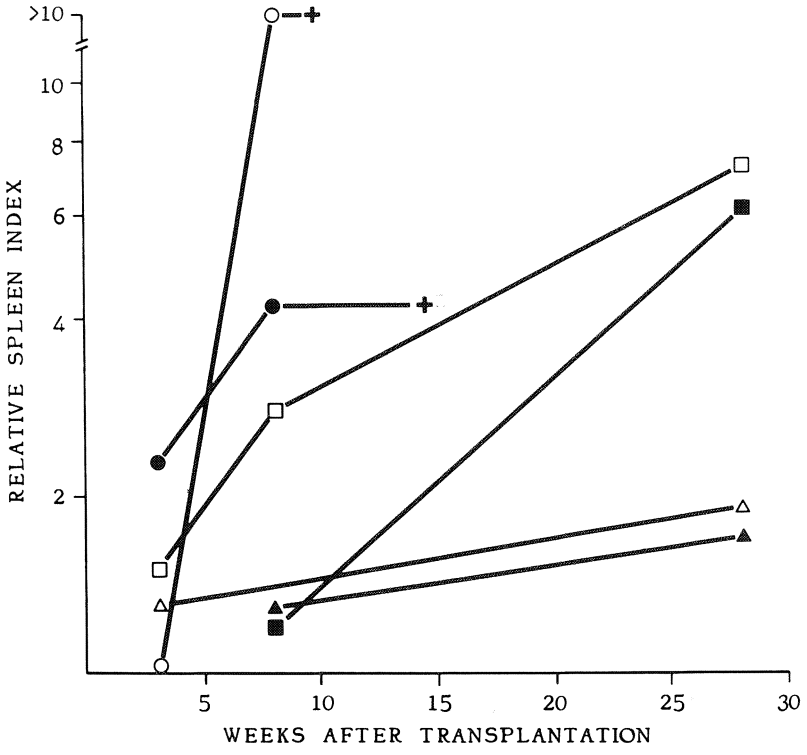


Fig. 5. Maturation of donor-derived GVH-reactive T cells in F_1 chimeras reconstituted with RATG-treated parental BM cells. 0.1 ml of 1:2 diluted whole blood from chimeras (see footnote to Figure 4) injected into (CH×B14)F_1 hybrid embryos. Values from 6 individual chickens (Lydyard and Ivanyi, 1975b)

blood. Analysis of chimeras 6 weeks after reconstitution with a RATG-treated BM graft showed adequate depletion of donor-derived GVH reactive cells and an unimpaired antibody response to immunization with SRBC (Fig. 4). However, a kinetic study of the peripheral blood lymphocytes (PBL) of chimeras up to 28 weeks after transplantation showed a gradual maturation of B14 anti-CH GVH-reactive cells in four out of six chickens (Fig. 5). Two chickens with the early onset of GVHR activity died whereas the remaining birds survived for at least 30 weeks after transplantation. These results demonstrate that maturation of T cells from parental-donor stem cells represents a serious limitation for the transplantation of T-cell deprived BM cells. The results are also in accord with studies in mice which showed maturation of GVH-reactive cells from Thy-1 negative progenitors within 2 weeks of transfer into irradiated syngeneic recipients (El-Arini and Osoba, 1973).

It is conceivable that chimeras which manifested a low anti-CH GVHR carried a high proportion of B14-donor derived T cells, while being specifically depleted of the anti-CH (host) reactive clone. This question was addressed by use of a discriminative GVHR assay in which B14 derived T cells reacting against third party (outbred) embryos were identified on the basis of their resistance to

treatment with anti-CH alloantiserum (Lydyard and Ivanyi, 1975b). Significant GVH activity resistant to inhibition with anti-CH alloantiserum was demonstrated in four chimeras (Table 3). Since the mechanism by which the anti-host reactive T cells are deleted, exhausted or tolerized is not understood, it is of further interest to examine by what means could we amplify such an apparently desirable outcome of allogeneic BM transplantation. The possible role of the host's thymic stroma (Yunis et al., 1974) and the MHC-determined restriction of cytotoxic T cells (Zinkernagel and Doherty, 1974) in these chimeras deserve further experimental work.

Table 3. Discriminative GVHR assay of PBL from B14 (T-depleted BM) → (CH×B14)F_1 lethally irradiated chimeras (Lydyard and Ivanyi, 1975b)

Lymphocyte donors	Recipient embryos:		
	(CH×B14)F_1 hybrid	Outbred	
		NCS	Anti-CH
Chimera No. 3	2.1	7.6	5.8
5	4.9	19.1	11.7
6	1.9	13.2	12.3
8	1.5	11.8	4.9
Host: (CH×B14)F_1	1.1	11.2	2.8
Donor: B14	8.5	8.5	9.0

8×10^5 PBL from 17 week old (F_1 hybrid assay at 8 or 28 weeks) chimeras were treated with 20% B14 anti-CH alloantiserum or NCS for 45 minutes at 37° C prior to injection into outbred embryos

E. Transplantation of Lymphoid Cells Between Allotype-congenic Strains

The failures of BM transplantation in man have been ascribed not only to lethal GVHR but also to graft rejection. The latter outcome may occur despite the use of HLA and mixed lymphocyte reaction (MLR) identical donor-host combinations. Current efforts are directed towards the search for unidentified minor histocompatibility antigens and also directed towards the characterization of resistance which may interfere with the engraftment of hematopoietic or lymphoid cells. Resistance (genetic, hybrid, allogeneic or hemapoietic) in mice is controlled by mechanisms which diverge from classical alloimmune reactions (Cudkowicz and Bennet, 1971; Lotzova, 1977) and is exercised probably by a subset of lymphoid cells which had been designated as natural killers (Kiessling et al., 1977; Hochman and Cudkowicz, 1977).

Transplantation of B cells between allotype congenic mouse strains is also restricted by resistance barriers (Klein and Herzenberg, 1967; Kobow and Weiler, 1975; Bosma et al., 1978). Histocompatible (B-locus homozygous) strains of chickens with distinct allotype markers have been established in two inbred lines (Ivanyi and Lydyard, 1975). The allotypes are controlled by two closely linked loci (M-1 and G-1) and the antigens are expressed in the constant

Table 4. Genetic background of chicken strains

Strains	MHC B-Locus	Ig allotype loci	
		M-1	G-1
B14A	14/14	a/a	g/g
B14B	14/14	a/a	a/a
B14C	14/14	b/b	i/i
B14D	14/14	b/b	g/g
CHA	12/12	a/a	g/g
CHB	12/12	a/a	a/a
(B14A×CHA)F_1	12/14	a/a	g/g

Change in the designation of G-1 locus controlled alleles: a → g, b → a, e → i (Foppoli et al., 1979)

region of μ and γ chains respectively (Ivanyi, 1975, 1978). The genotypes of chicken strains are shown in Table 4. The histocompatibility of allotype "congenic" B14 sub-strains has been tested by embryonal splenomegaly and MLR assays (Fig. 6, 7). The results showed strong reactions between the B14 and CH (B-locus incompatible) strains but a lack of significant stimulation between the allotype disparate substrains.

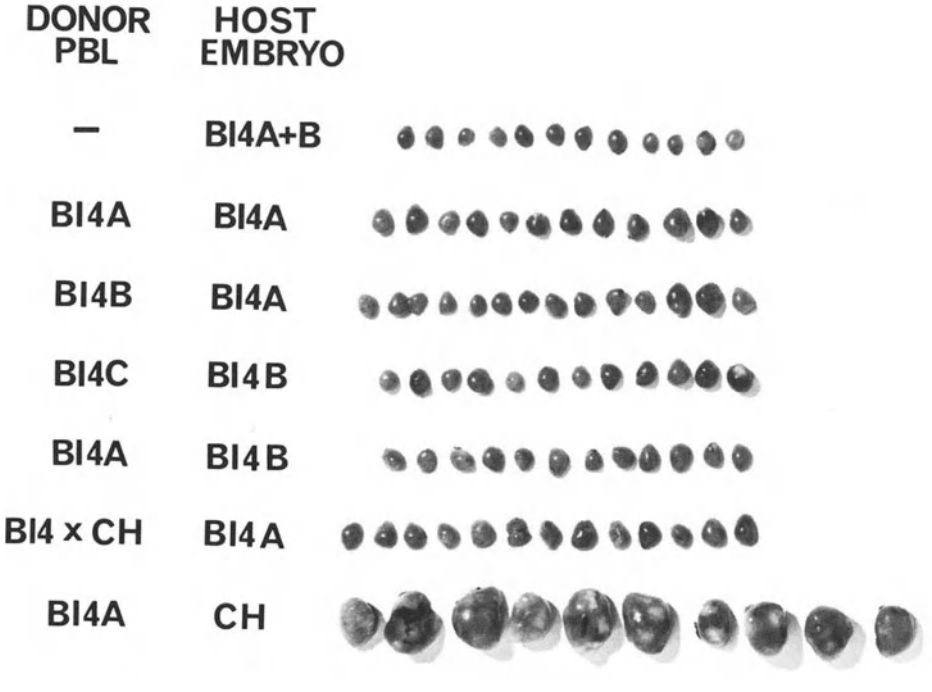


Fig. 6. Lack of GVHR splenomegaly reactions betweeen B14 substrains

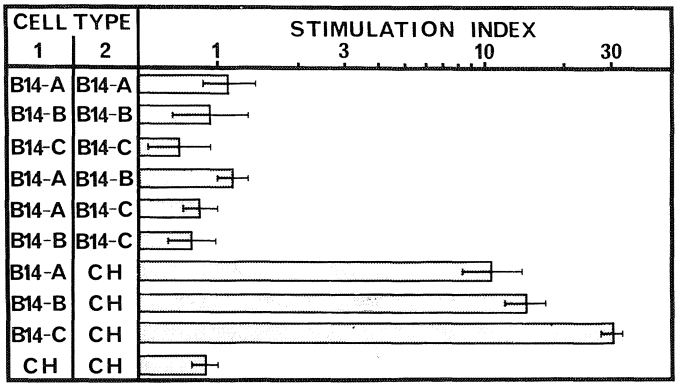


Fig. 7. Lack of mixed lymphocyte culture reactions between B14 substrains

Transplantation of lymphoid cells between the B14 sublines enables monitoring via the allotype markers of the relative contribution of donor and host derived B cells in chimeric chickens. Cell suspensions from various lymphoid organs of young adult (1–6 month old) donors were injected into juvenile (3–6 day old) recipients. Blood samples were drawn at various times after cell transfer and serum allotype levels were determined quantitatively by radial immunodiffusion in agar gels (Ivanyi and Makings, 1978).

Donor and host derived allotype levels were inversely related and determined by circumstances such as host age and conditioning and donor cell type and number (Table 5). An increase in the number of injected lymphoid cells resulted

Table 5. Requirements for breaking the host resistance

Age:	1>4>10 days
Drugs:	Cyclophosphamide> X-irradiation>none
Donor cell type:	Spleen>PBL>BM >thymus
Donor cell number:	$2-10\times10^7$

in higher donor allotype levels and suppression of host allotype synthesis (Fig. 8). Host conditioning with cyclophosphamide (CY) and γ-irradiation (^{137}Cs) was compared quantitatively and showed the following results. Three injections of 50 mg CY/kg produced little toxicity but complete reversal of host → donor allotypes (Fig. 9). On the other hand, γ-irradiation resulted in profound host allotype suppression only at doses which caused also high mortality of chickens. However, combined conditioning with CY and irradiation had a synergistic effect at doses which were ineffective alone (Fig. 10). The conditioning effect of CY was short-term since B cell engraftment declined when repopulation was delayed by 4–8 days (Table 6). In order to find out whether the higher efficacy of CY when compared with irradiation resulted from improved colonization of lymphoid organs, the homing of ^{51}Cr-labelled lymphocytes was determined 20 hours after injection (Table 7). The results indicated a significant decrease of radioactivity in

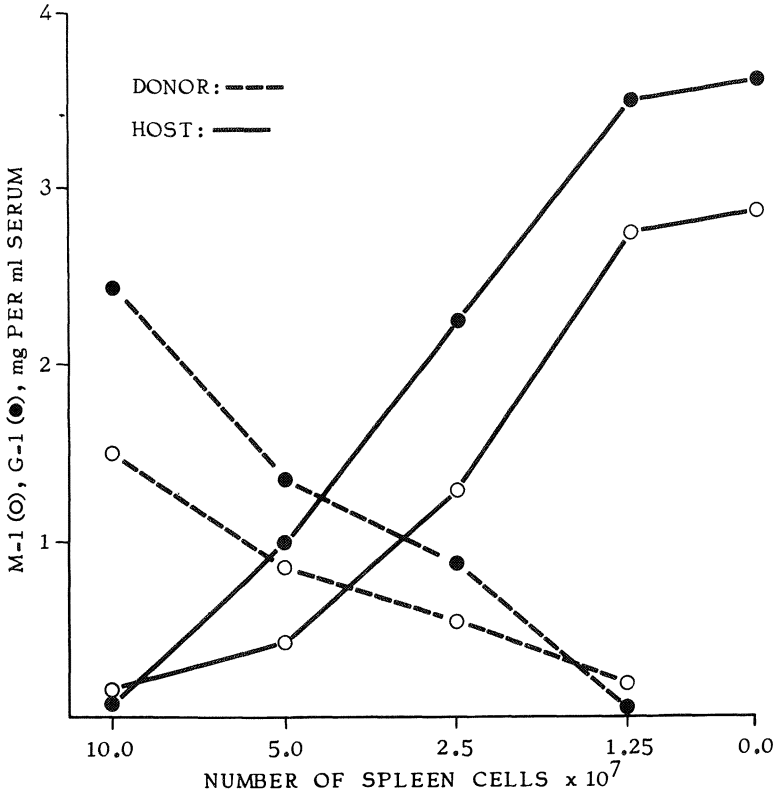


Fig. 8. Reciprocal relationship between donor- and host-derived allotypes in histocompatible chimeras. 2×10^7 PBL from B14A → B14B hosts (CY treated). Serum assay 2 weeks after cell transfer

Table 6. The effect of time delay in transferring cells after CY treatment

Interval between CY injection and cell transfer	Donor allotype synthesis			
	M-1		G-1	
	%+ve	μg/ml	%+ve	μg/ml
1 day	70	125	70	320
5 days	50	81	50	190
9 days	10	3	40	170

5×10^7 PBL from B14C → B14A. CY (120 mg/kg) at 4 days and assay 24 days after hatching

the bursa of Fabricius of CY-injected chickens and a relative increase of counts in the spleen. Homing of donor cells in the spleen combined with the selective suppression of host B cell differentiation by CY (Lerman and Weidanz, 1970) are apparently the favourable conditions required for establishing the B cell chimerism.

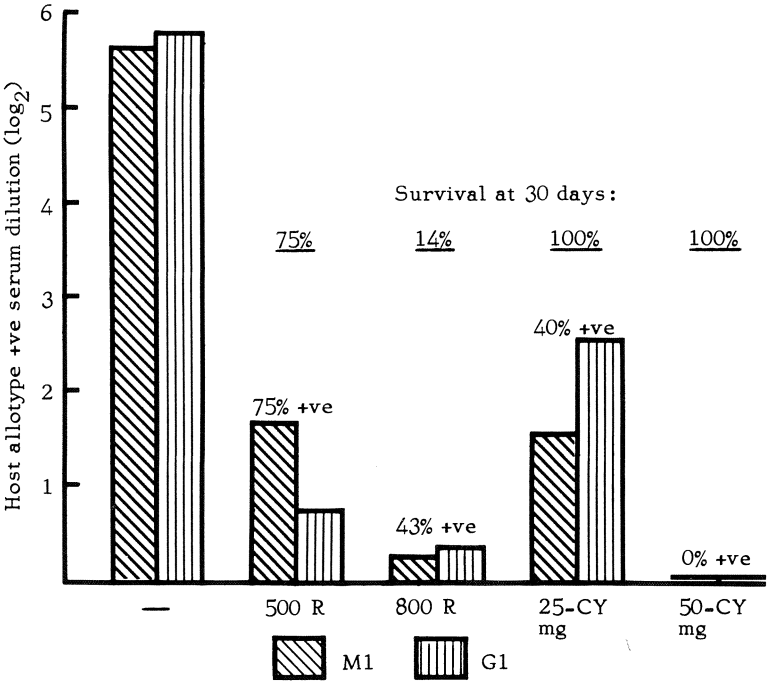


Fig. 9. Higher efficacy of sublethal doses of CY compared with γ-irradiation in promoting host allotype suppression. B14A, 5 day old hosts injected with 5×10^7 B14C PBL. CY injected on 3 consecutive days prior to cell transfer. All chickens were positive for donor derived allotypes. Assay 2 weeks after cell transfer

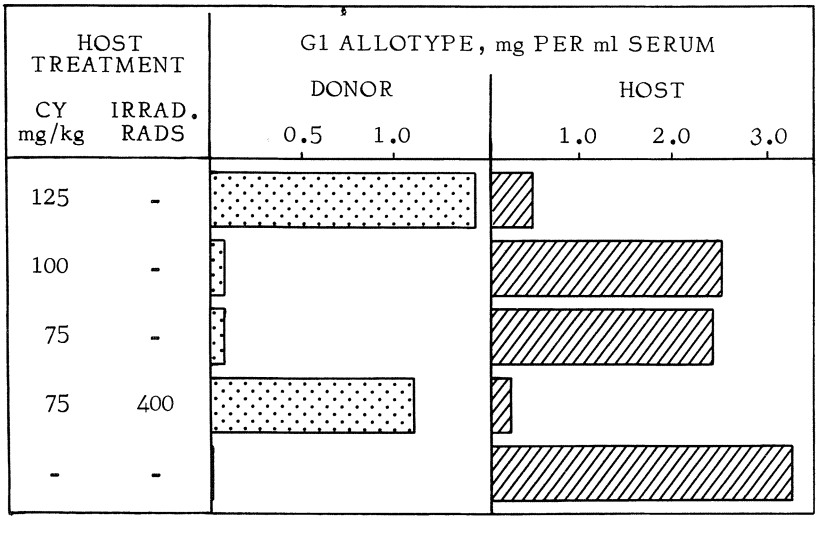


Fig. 10. Synergism between CY and irradiation in promoting B cell engraftment. 5×10^7 B14C spleen cells injected into 4 day old B14A recipients. Serum assay 3 weeks later

Table 7. Homing pattern of congenic spleen cells

Conditioning of recipients	CPM/organ		Weight mg		CPM/mg		Spleen/→ bursa
	Bursa	Spleen	Bursa	Spleen	Bursa	Spleen	
None	142 ±6.9	2005 ±91.9	92	20.3	1.54	98.8	64.1
300R	128 ±8.9	1204 ±71.5	82	14.7	1.56	81.9	52.5
600R	122 ±4.7	1164 ±101.2	102	11.7	1.20	99.5	82.9
5 mg CY	81.9 ±24.2	1844 ±238.4	97	14.4	0.84	128.0	152.4
10 mg CY	71.7 ±7.8	1692 ±71.3	95	13.7	0.74	123.5	166.2

B14 chickens (5–6 per group) were conditioned 3 days after hatching and injected iv. with 1×10^7 ^{51}Cr labelled B14C spleen cells (43×10^3 cpm) 1 day later. Organs were harvested after 20 hours

To determine the nature of donor effector cells which produced suppression of host allotype synthesis, we injected CY-treated hosts with purified T lymphocytes (Fig. 11). An absence of donor allotypes in the sera of recipient chickens was taken as evidence for the complete depletion of B cells from the fractionated cell suspension. Under such conditions T cells unequivocally failed to inhibit host serum allotype levels. Furthermore, mixed transfer of T cells with limiting doses of whole spleen cells failed to amplify donor allotype levels or the inhibition of host allotype synthesis. In view of these results it seems unlikely that alloimmunity (which would have been mediated by T cells) against minor histocompatibility antigens may play a role in the mechanism of host allotype suppression.

Immunity against M-1 allotype which is expressed on the surface of B cells (Ivanyi and Hudson, 1979; Ratcliffe and Ivanyi, 1979) was considered as an alternative mechanism but this seems unlikely since: a) passive injection of serum which was homologous with the host failed to compete with host allotype suppression; b) host G-1 allotype suppression occurred also in the B14A → B14B strain combination in which M-1a is shared by both strains; c) sera from suppressed chickens failed to manifest any anti-allotype activity (Ivanyi and Makings, 1978).

In conclusion we suggest that host resistance towards transplanted histocompatible B cells and the suppression of host B cells in chimeras result from a two-way antagonistic interaction between the respective B cell populations (Ivanyi and Makings, 1978, 1979). The proposed non-immune surveillance mechanism may represent a primitive form of regulation by which aberrant cells can be eliminated. Under experimental conditions when B cells are compromised by an immunosuppressive agent such as CY, non-suppressed chimeric B cells may interfere with subsequent recruitment from immature precursors. It seems plausible that resistance towards cells of diverse histogenetic origin is mediated by distinct mechanisms possibly related to the type and tissue distribution of

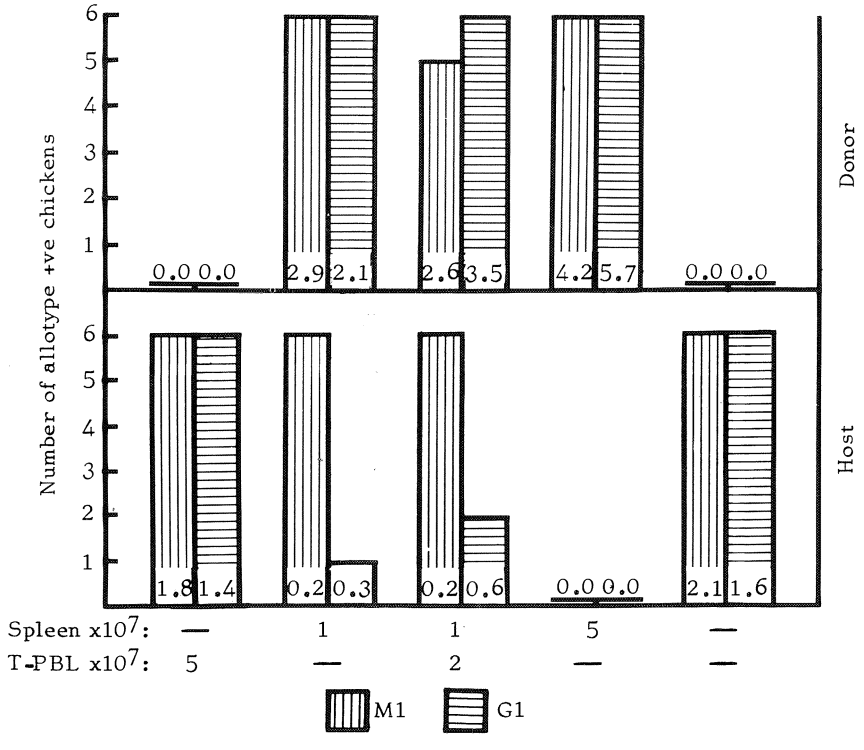


Fig. 11. Failure of histocompatible T cells to suppress host allotype synthesis. B14C, CY-conditioned recipients injected with B14A spleen and PBL-T cells. Assay 3 weeks after cell transfer. Numbers inside columns: mg M-1 or G-1 per ml serum. B cells were depleted by a modified EA-RFC separation technique (Ivanyi and Makings, 1978)

homologous host cells. This view is supported by recent data demonstrating that the homing of chimeric cells in CBA/N mice is selective for those lymphoid organs which are genetically deficient for the corresponding B cell subset (Volf et al., 1978). Alternatively, the role of a common subset of lymphoid cells such as natural killers cannot be excluded.

F. Prolongation of B Cell Chimerism by a Chronic GVHR

Resistance of juvenile chickens against engraftment of histocompatible B cells has been ascribed in the previous paragraph to a mechanism similar to that which operates the host resistance towards hemopoietic or tumour cells. This view is further supported by the finding of even greater resistance when transplanting parental B cells into F_1 hybrid recipients (Ivanyi and Makings, 1979). Donor and host derived allotype markers were monitored in the sera of B14C (M-1b, G-1i)→(B14A×CHA)F_1 (M-1a, G-1g) chimeras. Whereas in histocompatible pairs the difference in the efficacy of conditioning with CY or irradiation was only quantitative, in the semiallogeneic combination, sub-lethal

Donor cells	Host conditioning	Survival	Ig allotype
Spleen	None	>70 days	Host
	600R	<20 days	Host
	120 mg/kg CY	<20 days	Donor
BM	None	>70 days	Host
	600 R	>70 days	Host
	120 mg/kg CY	>70 days	Donor

Transfer of 5×10^7 B14C cells into 5 day old (B14A-×CHA)F_1 hybrid hosts. Assay 2 weeks after cell transfer

Table 8. Parental B cell chimerism in CY-conditioned but not in sublethally irradiated F_1 hybrid hosts

(600 rads) irradiation completely failed to promote B-cell engraftment. However, donor allotypes were detected in hosts which had been conditioned with 120 mg CY/kg (Table 8). The results also indicated that the resistance of F_1 hybrid hosts is weaker towards parental T than against B cells. Thus, irradiation of hosts with 600 or 300 rads prior to injection of parental spleen cells resulted in lethal GVHR which did not occur in untreated recipients.

Repopulation of CY-treated F_1 hosts showed both allotype chimerism and prolonged survival. Comparison of these semiallogeneic chimeras with the allotype-congenic histocompatible chimeras revealed some surprising differences

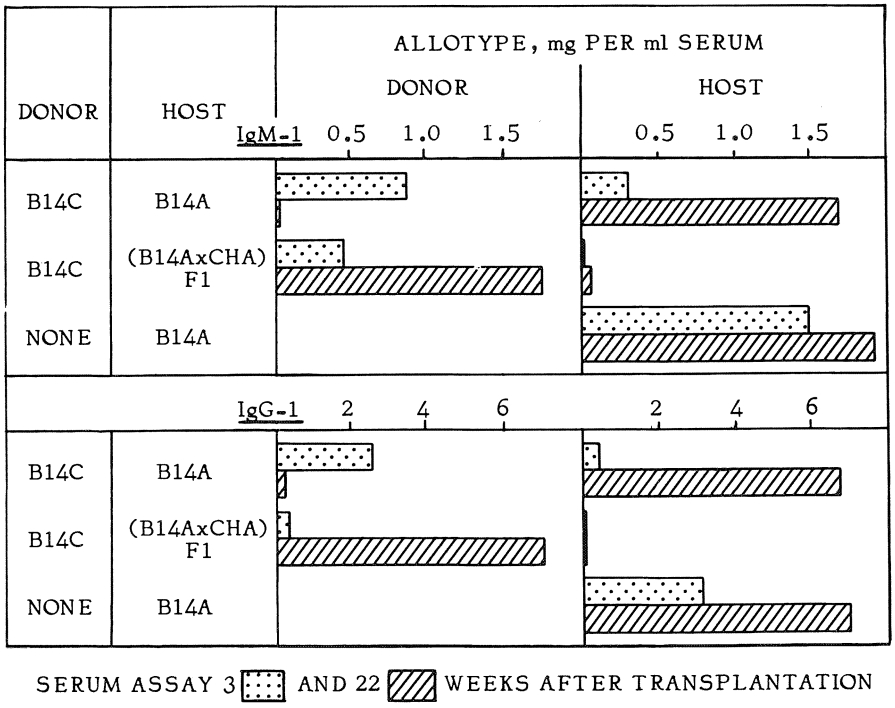


Fig. 12. Long-term B-cell chimerism in B14C → (B14A×CHA)F_1 hybrid but not in B14C → B14A chimeras. 5×10^7 BM cells injected into CY-conditioned 4 day old hosts

(Fig. 12). Whereas histocompatible chimeras rejected their B cell graft and recovered host-derived allotype levels between 3 and 22 weeks after repopulation, semiallogeneic chimeras persisted in producing donor allotypes for at least 6 months. The serum concentrations of chimeric M-1b and G-1i allotypes varied and reached abnormally high levels in some birds, although host allotype levels were uniformly suppressed (Table 9). The simplest explanation of these results is that an ongoing chronic GVHR impaired the recruitment of host B cells and hence abrogated the "displacement" of grafted B cells. The finding of bursal hypoplasia in embryonal GVHR (see Sect. B) suggests that stem cells or B cell precursors are indeed very vulnerable targets for alloimmune lymphocytes. Furthermore, the GVHR by its "allogeneic effect" (Kreth and Williamson, 1971; Osborne and Katz, 1972), could have further amplified the proliferation or the rate of Ig synthesis by donor B cells. Despite high donor allotype levels, most of the chimeric chickens failed to produce antibodies after injection with SRBC (Table 9). Thus, GVHR resulted in paradoxic effects: on the one hand it promoted long-term Ig-secreting chimeric B cells, on the other it suppressed the antibody response to active immunization.

Table 9. Immunosuppression in donor allotype secreting semiallogeneic chimeras

Chicken No.	Donor BM 5×10^7	Allotype mg/μl				Anti-SRBC $\log_2$ titer	
		Donor		Host			
		M-1b	G-1i	M-1a	G-1a	–	ME[a]
515	+	5.3	11.5	<0.1	0.3	7	0
521	+	2.5	3.0	<0.1	<0.1	0	0
525	+	1.2	12.2	<0.1	<0.1	0	0
520	+	<0.1	2.9	<0.1	0.1	8	5
534	+	3.2	4.0	<0.1	0.1	0	0
519	+	0.1	3.3	<0.1	0.6	0	0
531	–	–	–	1.2	2.0	9	6
539	–	–	–	1.2	4.0	13	5
514	–	–	–	0.8	6.1	11	4

[a] 0.2M mercaptoethanol treatment 1 hour at 37° C
Transplantation B14C → (B14A×CHA)F_1 (CY-conditioned, 4 day old). Assay 6 months after repopulation

Another aspect of these experiments may concern the potential risk of using T cell deprived BM for clinical transplantation. It appears that chimeric B cells are subject to greater risk of protracted rejection in the absence of any GVHR. This rejection is probably due to non-immune surveillance by the newly recruited host's own B cells, and therefore difficult to control. Further use of MHC and allotype defined chicken strains seems highly suitable for experiments along these lines.

References

Albini, B., Wick, G.: Delineation of B and T lymphoid cells in the chicken. J. Immunol. *112*, 444–450 (1974)

Blomgren, H., Andersson, B.: Inhibition of erythroid cell growth in irradiated mice by allogeneic lymphoid cells: A quantitative method for graft-versus-host-reactivity of lymphoid cells. Cell. Immunol. *3*, 318–325 (1972)

Bosma, M. J., Bosma, G. C., Owen, J. L.: Prevention of immunoglobulin production by allotype-dependent T cells. Eur. J. Immunol. *8*, 562–568 (1978)

Brochier, J., Abou-Hamed, Y. A., Guehos, J. P., Revillard, J. P.: Study of human T and B lymphocytes with heterologous antisera. Immunology *31*, 749–765 (1976)

Chanard, J., Bach, J. F., Assailly, J., Funck-Brentano, J.-L.: Hepatic homing of labelled lymphocytes in man. Br. Med. J. *2*, 502–504 (1972)

Cudkowicz, G., Bennett, M.: Peculiar immunobiology of bone marrow allografts. I. Graft rejection by irradiated responder mice. J. Exp. Med. *134*, 83–102 (1971)

Davis, W. E., Cole, L., Schaffer, W. T.: Graft-versus-host reactions in non-irradiated mice. Early suppression of Jerne plaques and hemopoietic colony-forming units. Transplantation *9*, 529–535 (1970)

El-Arini, M. O., Osoba, D.: Differentiation of thymus-derived cells from precursors in mouse bone marrow. J. Exp. Med. *137*, 821–837 (1973)

Feiglova, E., Pichlikova, L., Nouza, K.: Antilymphocyte activity of rabbit anti-chicken anti-brain sera. Folia biol. (Praha) *18*, 256–263 (1972)

Foppoli, J. M., Ch'ng, L. K., Benedict, A. A., Ivanyi, J., Derka, J., Wakeland, E. K.: Genetic nomenclature for chicken immunoglobulin allotypes: an extensive survey of inbred lines and antisera. Immunogenetics *8*, 385–404 (1979)

Forget, A., Potworowski, E. F., Richer, G., Borduas, A. G.: Antigenic specificities of bursal and thymic lymphocytes in the chicken. Immunology *19*, 465–468 (1970)

Galton, J.: Differentiation alloantigens in the chicken. Ph.D. Thesis, University of London 1978

Greaves, M. F., Tursi, A., Playfair, J. H. L., Torrigiani, G., Zamir, R., Roitt, I. M.: Immunosuppressive potency and in vitro activity of antilymphocyte globulin. Lancet *1969 I*, 68–72

Hochman, P. S., Cudkowicz, G.: Different sensitivities to hydrocortisone of natural killer cell activity and hybrid resistance to parental marrow grafts. J. Immunol. *119*, 2013–2015 (1977)

Houssaint, E., Belo, M., Le Douarin, N. M.: Investigations on cell lineage and tissue interactions in the developing bursa of Fabricius through interspecific chimeras. Dev. Biol. *53*, 250–264 (1976)

Hudson, L., Roitt, I. M.: Immunofluorescent detection of surface antigens specific to T and B lymphocytes in the chicken. Eur. J. Immunol. *3*, 63–67 (1973)

Ivanyi, J.: Polymorphism of chicken serum allotypes. J. Immunogenet. *2*, 69–78 (1975)

Ivanyi, J.: Recombination of C_H genes encoding the M1 (IgM) and G1 (IgG) chicken allotypes. Nature *272*, 166–167 (1978)

Ivanyi, J., Hudson, L.: Allelic exclusion of M1 (IgM) allotype on the surface of chicken B cells. Immunology *35*, 941–945 (1979)

Ivanyi, J., Lydyard, P.: Delineation of chicken lymphocyte populations by specific anti-thymus and anti-bursa sera. Cell. Immunol. *5*, 180–189 (1972)

Ivanyi, J., Lydyard, P.: Segregation of allotypes in a strain of chickens homozygous for the B locus. Immunogenetics *2*, 285–289 (1975)

Ivanyi, J., Makings, C. W.: Antagonism between donor and host B cells in allotype congenic chicken chimeras. Transplantation *26*, 221–227 (1978)

Ivanyi, J., Makings, C. W.: Allotype analysis of B cell chimeric chickens. Transplantation (in press)

Kadowaki, J. I., Zuelzer, W. W., Brough, A. S., Thompson, R. I., Wooley, P. V., Gruber, D.: XX/XY lymphoid chimerism in congenital immunological deficiency syndrome with thymic alymphoplasia. Lancet *1965 II*, 1152 (1965)

Kiessling, R., Hochman, P. S., Haller, O., Shearer, G. M., Wigzell, H., Cudkowicz, G.: Evidence for a similar or common mechanism for natural killer cell activity and resistance to hemopoietic grafts. Eur. J. Immunol. *7*, 655–663 (1977)

Klein, J., Herzenberg, L. A.: Congenic mouse strains with different immunoglobulin allotypes. I. Breeding scheme, histocompatibility tests, and kinetics of γG_{2a}-globulin production by transferred cells for C3H.SW and its congenic partner CWB/5[1]. Transplantation *5*, 1484–1495 (1967)

Kobow, U., Weiler, E.: Permissiveness of athymic ("nude") mice towards congenic memory cells. Eur. J. Immunol. *5*, 628–632 (1975)

Kreth, H. W., Williamson, A. R.: Cell surveillance model for lymphocyte co-operation. Nature *234*, 454–456 (1971)

Lerman, S. P., Weidanz, W. P.: The effect of cyclophosphamide on the ontogeny of the humoral immune response in chickens. J. Immunol. *105*, 614–619 (1970)

Lotzova, E.: Resistance to parental, allogeneic and xenogeneic hemopoietic grafts in irradiated mice. Exp. Hematol. *5*, 215–235 (1977)

Lydyard, P., Ivanyi, J.: Suppression of graft-versus-host-reactive lymphocytes by heterologous anti-thymus serum *in vitro*. Transplantation *12*, 493–499 (1971)

Lydyard, P. M., Ivanyi, J.: The role of opsonization in antithymocyte globulin-induced suppression of graft-versus-host reaction in chick embryos. Transplantation *17*, 400–404 (1974)

Lydyard, P. M., Ivanyi, J.: Immunodeficiency in the chicken. III. Hypoplasia of bursal follicles following intravenous injection of embryos with lipopolysaccharide or allogeneic lymphocytes. Immunology *28*, 1023–1031 (1975a)

Lydyard, P., Ivanyi, J.: Chimerism of immunocompetent cells in allogeneic bone marrow-reconstituted lethally irradiated chickens. Transplantation *20*, 155–162 (1975b)

McArthur, W. P., Chapman, J., Thorbecke, G. J.: Immunocompetent cells of the chicken. I. Specific surface antigenic markers on bursa and thymus cells. J. Exp. Med. *134*, 1036–1045 (1971)

Miggiano, V. C., Birgen, I., Pink, J. R. L.: The mixed leukocyte reaction in chickens. Evidence for control by the major histocompatibility complex. Eur. J. Immunol. *4*, 397–401 (1974)

Moore, M. A. S., Owen, J. J. T.: Stem-cell migration in developing myeloid and lymphoid systems. Lancet *1967 II*, 658–659

Mosedale, B., Smith, M. A., Courtenay, J. S.: Preparation and characterization of antithymocyte serum and globulin without stem cell activity. Transplantation *22*, 122–131 (1976)

Nisbet, N. W., Simonsen, M., Zaleski, M.: The frequency of antigen-sensitive cells in tissue transplantation. J. Exp. Med. *129*, 459–467 (1969)

Osborne, D. P., Katz, D. H.: The allogeneic effect in inbred mice. I. Experimental conditions for the enhancement of haptenspecific secondary antibody responses by the graft-versus-host reaction. J. exp. Med. *136*, 439–454 (1972)

Owen, J. J. T., Moore, M. A. S., Harrison, G. A.: Chromosome marker studies in the graft-versus-host reaction in the chick embryo. Nature *207*, 313–315 (1965)

Potworowski, E. F., Zavallone, J. D., Gilker, J. C., Lamoureux, G.: Inhibition of the graft-versus-host reaction by thymus-specific antibodies. Rev. Europ. Etudes Clin. et Biol. *16*, 155–157 (1971)

Ratcliffe, M., Ivanyi, J.: Allelic exclusion of surface IgM allotypes on spleen and bursal B cells in the chicken. Immunogenetics *9*, 149–156 (1979)

Schierman, L. W., Nordskog, A. W.: Influence of B blood group-histocompatibility locus in chickens on a graft-versus-host reaction. Nature *197*, 511–512 (1963)

Simonsen, M.: The impact on the developing embryo and newborn animal of adult homologous cells. Acta Path. Microbiol. Scand. *40*, 480–500 (1957)

Smith, R. W., Terry, W. D., Buell, D. N., Sell, K. W.: An antigenic marker for human thymic lymphocytes. J. Immunol. *110*, 884–887 (1973)

Taub, R. N., Lance, E. M.: Effects of heterologous anti-lymphocyte serum on the distribution of ^{51}Cr-labelled lymph node cells in mice. Immunology *15*, 633–642 (1968)

Touraine, J. L., Touraine, F., Kiszkiss, D. F., Choi, Y. S., Good, R. A.: Heterologous specific antiserum for identification of human T lymphocytes. Clin. Exp. Immunol. *16*, 503–520 (1974)

Volf, D., Sensenbrenner, L. L., Sharkis, S. J., Elfenbein, G. J., Scher, I.: Induction of partial chimerism in nonirradiated B-lymphocyte-deficient CBA/N mice. J. Exp. Med. *147*, 940–945 (1978)

Walker, K. Z., Lafferty, K. J., Schoefl, G. I.: Pathogenesis of the graft-versus-host reaction in chicken embryos. Requirement of yolk sac-derived stem cells for the development of proliferative lesions. Aust. J. Exp. Biol. Med. Sci. *51*, 347–355 (1973)

Weber, W. T.: Analysis of host and donor cell proliferation in chorioallantoic pocks. Transplantation *10*, 275–277 (1970)

Wick, G., Albini, B., Milgrom, F.: Antigenic surface determinants of chicken lymphoid cells. I. Serologic properties of anti-bursa and anti-thymus sera. Clin. Exp. Immunol. *15*, 237–249 (1973)

Wick, G., Steiner, R., Wolner, E., Ahmad, R., Zeilinger, M., Mittermayer, K., Stacher, A.: Production and diagnostic application of anti-human T-cell antisera. Postgrad. Med. J. *52*, Suppl. 5, 20–26 (1976)
Yunis, E. J., Good, R. A., Smith, J., Stutman, O.: Protection of lethally irradiated mice by spleen cells from neonatally thymetomized mice. Proc. Natl. Acad. Sci. USA *71*, 2544–2548 (1974)
Zinkernagel, R. M., Doherty, P. C.: Immunological surveillance against altered-self components by sensitized T lymphocytes in lymphocytic choriomeningitis. Nature *251*, 547–548 (1974)
Zola, H.: Preparation of antisera with specificity for human T lymphocytes. Transplantation *23*, 222–229 (1977)

Discussion

Müller-Ruchholtz: Do you depend on a certain timing of grafting, irradiation and cyclophosphamide application?
Ivanyi: We could engraft the B-cells until 5 to 7 days after cyclophosphamide. The host had regained its resistance if the graft was given later. In congenic combinations the resistance could be overcome by injecting more cells. In F_1-hybrids the resistance was much greater and required cyclophosphamide for suppression.
Halle-Pannenko: Is there a competition for restoration if donor and host cells are both present in the inocculum?
Ivanyi: That would require three markers. We have not done this experiment although we have the model which would enable to do that.
Halle-Pannenko: We have observed that the addition of isogenic cells to the bone marrow inocculum decreased the GVH reaction, but we do not know whether this was due to the preferential restoration of the isogenic cells.

5 Suppressions of Graft Versus Host Reactions with Cyclosporin A

Use of Cyclosporin A (CsA) in a Rat Model of Allogeneic Marrow Transplantation*

P. J. Tutschka, W. E. Beschorner, and A. D. Hess

A. Introduction

Allogeneic bone marrow transplantation has assumed a definitive therapeutic role for the treatment of severe aplastic anemia and severe combined immunodeficiency diseases and is considered the per primum therapy for these disorders, provided that a donor is available who is genotypically HL-A identical to the prospective recipient (Santos, 1979; Thomas et al., 1975; Tutschka et al., 1977a). The ultimate role of allogeneic BMT for the treatment of malignant diseases, especially lympho-hematopoietic malignancies, has not been established although this measure is considered a management option for patients with acute leukemia (Tutschka et al., 1978; Tutschka et al., 1979; Santos et al., 1976).

The reported long term success rates of not more than 40–50% for marrow transplants in aplastic anemia and only 10–20% in acute leukemia indicate that several serious complications prevent the full realization of the therapeutic potential of this procedure (Tutschka et al., 1978, Tutschka et al., 1979).

To obtain allogeneic engraftment, patients, even those treated for aplastic anemia, have to be conditioned with cytoreductive agents like cyclophosphamide (Cy) or total body irradiation (TBI), agents that have relatively low therapeutic indices for immunosuppression and marked associated toxicities (Santos et al., 1976; Lichter et al., 1979).

Despite using high doses of immunosuppressive agents with their inherent toxic side effects allogeneic marrow grafts often fail to take, a problem that is of particular importance in the treatment of aplastic anemia (Tutschka et al., 1977a; Storb et al., 1978; Elfenbein et al., 1978). In this situation up to 48% of patients conditioned with high doses of Cy and grafted with allogeneic marrow fail to engraft, presumably because the recipients have been sensitized to minor histocompatibility antigens of the prospective donor (Storb et al., 1970; Tutschka and Santos, 1975a).

The central problem of allogeneic bone marrow transplantation is graft-versus-host disease (GVHD) in its acute and chronic forms (Grebe and Streilein, 1976). After successfully engrafting, patients, even if matched with their respective donors at the major histocompatibility complex and given post-transplant immunosuppressive agents in an attempt to prophylaxe GVHD, develop GVHD in a high incidence rate of reported 50–70%, which is fatal in up to 25%.

---

* Supported by ACS grant No. Ch-113, ACS Institutional Research Grant No. IN-11P and CA-15396

Related to the preparative regimen and to the immunologic complex "GVHD" is a syndrome of immunodeficiency that occurs shortly after transplantation, is initially expressed as a severe combined immunodeficiency but later develops into a T-dependent immunodeficiency (Elfenbein et al., 1976; Noel et al., 1978; Gale et al., 1978).

This immune defect is considered a major factor contributing to the high susceptibility of transplant recipients toward opportunistic infections, especially those leading to interstitial pneumonia, a complication with a high incidence (30–50%) and extreme high mortality rate (50–70% of those afflicted with the syndrome) (Neiman et al., 1977; Khouri et al., 1979).

Attempts to overcome these complications have been largely unsuccessful, in part, because new immunosuppressive principles for better pretransplant conditioning and post-transplant therapy were not available.

Cyclosporin A (CsA), an undecapeptide derived from certain fungi imperfecti (Rüegger et al., 1976; Petcher et al., 1976) (Cylindrocarpon lucidum Booth and Trichoderma polysporum Rifai) has been reported to have very profound immunosuppressive properties ranging from inhibition of plaque forming cells and hemagglutinin formation to suppression of delayed type hypersensitivity skin reactions, skin graft rejection and experimental allergic encephalomyelitis in various animal systems (Borel et al., 1976a; Borel, 1976b).

The remarkable immunosuppressive activity of CsA was associated with minimal if any effects on the hematopoietic tissues (Borel et al., 1977). Moreover, CsA appeared to be capable of inducing transplantation tolerance in several animal models, since orthotopic renal and cardiac allografts from donors mismatched at the major histocompatibility complex (MHC) were retained indefinitely and in excellent health even after the administration of CsA had been discontinued (Calne et al., 1978; Green and Allison, 1978).

It seemed resonable to study the effects of this new immunosuppressive agent in an animal model of bone marrow transplantation, hoping that this drug might be capable of counteracting graft-versus-host disease and accelerating the immune recovery of allogeneic chimeras post transplantation. This study will report about some preliminary results of CsA in a rat bone marrow transplantation model.

B. Materials and Methods

I. Animals

Female Lewis (Ag-B1), ACI (Ag-B4) and BN (Ag-B3) rats, 10–12 weeks old were obtained from Microbiological Associates, Bethesda, Maryland. Animals were housed in polycarbonate cages, four to a cage. They were provided with tap water and Purina chow ad libitum.

II. Drugs and Irradiation

Busulfan (BU) was prepared in 2.5% carboxymethyl cellulose in water; all injections were given i.p. a few minutes after preparation of the drug in a volume of 10 mg/kg of bodyweight.

Cyclophosphamide (CY) was prepared in saline and injected intraperitoneally in a volume of 10 mg/kg of bodyweight.

Rabbit-anti-rat serum (RARTS) was prepared using the method described previously (Tutschka et al., 1975a). This ATS when given IP in six consecutive daily doses of 1.5 ml to Lewis rats, prolonged the mean survival of AgB incompatible skin grafts to 24.8 ± 1.82 days compared with a mean survival of 11.0 ± 1.7 days using normal rabbit serum.

A dual source ^{137}Ce small animal irradiator delivering 136 rads/min was used for total body irradiation of the recipients as well as to irradiate test cells.

CsA was generously supplied by Drs. Wiskott and Lardi from Sandoz, LTD. It was supplied to us in vials as an oily emulsion. To dilute the drug to the appropriate final concentration, the content of the vials was warmed to 37° C, and the content diluted in warm saline with addition of 5% fresh Lewis serum as stabilizer.

III. Preparation of Marrow Cell Inocula and Assessment of Chimerism

Preparation of marrow cell inocula was done as described previously (Tutschka and Santos, 1975b). Antisera specific for ACI immunoglobulin allotype was used in a gel-diffusion test to assess lymphoid chimerism indirectly as described before (Humphrey and Santos, 1971). The peripheral blood cells of ACI-Lewis (donor-host) chimeras were typed with strain-specific cytotoxic isoantisera (Tutschka and Santos, 1977b) to assess completeness of chimerism.

In addition, animals surviving 28 days were implanted with 1-cm circular skin grafts from the donor, recipient, and a third party (BN) strain by techniques described previously (Santos and Owens, 1965).

The clinical course of the animals was followed by daily observation and periodic weight determinations as described before. At certain specified intervals as well as on all animals dying in the course of the experiment autopsies were performed and histological lesions characteristic of graft-versus-host reactions were recorded as previously described (Tutschka and Santos, 1975b).

IV. Contact Sensitization to 2–4 Dinitrochlorobenzene (DNCB)

Rats were anesthetized with chloral hydrate, the hair on the abdomen was removed with an electric hair cutter, the abdominal skin cleansed with acetone and the DNCB solution applied to the abdomen with a micropipette spreading the DNCB over an area of approximately 1 cm^2. Fourteen days after applying the sensitizing dose (660 μg, right side of abdomen) a challenging dose was applied (16 μg, left side of abdomen) and the site of the challenging dose biopsied after another 48 hours. The histologic reaction was graded on a scale from I to IV as described previously (Slavin et al., 1973).

V. Immunization with Sheep Red Blood Cells (SRBC)

Immunization with SRBC and determination of saline agglutination and 2-ME resistant antibody titers were performed as described previously (Santos and Owens, 1966).

VI. Mixed Lymphocyte Culture Techniques

1. Depletion of Macrophages

Since the spleen cells from normal rats give erratic results in a MLC, probably due to naturally occuring suppressor cells of presumed macrophage origin, we used a method modified after Weiss and Fitch to remove macrophages (Weiss and Fitch, 1977). Briefly, all the spleen cells used in the MLC are incubated with carbonyl iron, and the iron containing cells are removed with a powerful magnet. This procedure allows the recovery of 65–75% of the original cell prepartion.

2. Mixed Lymphocyte Culture

Spleen cells, after depleting them of iron containing cells, are suspended in RMPI 1640 supplemented with 5% heat inactivated fresh BN serum, 5×10^{-5} M 2-Mercaptoethanol (2-ME), 0.25 μg/ml glutamine, penicillin (50 U/ml) and streptomycin (50 mcg/ml). Five $\times 10^6$/ml responding cells are cocultured with 6×10^6/ml stimulating cells in U-bottom microtiter plates (Cooke) for five days at 37° C in a humidified atmosphere of 5% CO_2 in air. Thereafter, 1 μCi of ^3H-thymidine is added and another 18 hours incubation performed prior to counting in a liquid scintillation counter. Results are expressed as mean cpm ± SD from triplicate cultures. To assess for suppression of the MLC macrophage depleted splenocytes from chimeras or normal ACI donors are added at day 0 of the culture.

C. Results

I. Use of CsA as Conditioning Agent for Marrow Transplantation Across the MHC (Table 1)

Groups of Lewis rats, eight per group, were given 30 mg/kg BU alone (groups 1, 2, 3) to together with 150 mg/kg CY (group 5), a short course of RARTS i.p. (RARTS 1.5 ml/day daily from day −8 to day −2, group 4) or CsA (group 6). CsA was administered daily subcutaneously from day −8 to day +18 in a dose of 25 mg/kg/day. One day after injecting BU 60×10^6 nucleated marrow cells were given intravenously. Lewis rats given a lethal dose of BU died of marrow failure (group 1), unless protected by syngeneic marrow (group 2). ACI marrow was not accepted by Lewis animals prepared with BU alone (group 3). Animals that were in addition to BU given adequate immunosuppression with either CY, RARTS or CsA engrafted and showed complete chimerism as documented by allotype analysis and by typing of peripheral lymphocytes. Of note is that no mortality was observed in the CsA treated group during the first 28 days.

II. Use of CsA to Abolish Sensitization to Major Histocompatibility Antigens (Table 2)

Lewis rats, myelosuppressed with a lethal dose of BU (30 mg/kg) and conditioned with CY (150 mg/kg) and CsA (25 mg/kg daily s.c.) readily accepted a histoincompatible ACI marrow graft (group 1). Sensitizing the recipients by injecting 10^7 spleen cells from the ACI donor i.v. several days prior to the conditioning resulted in a failure to engraft (group 2), and neither addition of CsA post transplantation (group 3) nor immediately after sensitization (group 4) permitted engraftment. Restimulation of the recipients with donor antigen immediately prior to administering CsA was not successful either in abolishing sensitization (group 6).

III. Use of CsA to Prevent Graft-Versus-Host Reaction (Table 3)

Lewis rats, 16 per group, were given 1,000 rads TBI and injected with 60×10^6 nucleated syngeneic Lewis or allogeneic, strongly histoincompatible ACI marrow cells (groups 1 and 3). Similarly prepared Lewis recipients, in addition, were given 25 mg/kg CsA s.c. daily for 18 days starting one day after marrow transplantation (groups 2 and 4). Two representative animals out of each group were sacrificed on days 21, 28, and 56 and examined histologically for the presence of graft-versus-host disease. Acute, severe, and ultimately fatal GVHD was seen in the allogeneic group not receiving CsA (group 3). Administration of CsA for 18 days completely prevented GVHD clinically and histologically (group 4).

IV. Use of CsA to Treat Graft-Versus-Host Disease (Table 4)

Groups of Lewis rats were conditioned with 1,000 rads TBI and grafted with 60×10^6 ACI marrow cells. Post-transplantation 25 mg/kg CsA was given s.c.

Table 1. Use of cyclosporin (CsA) as an immunosuppressant to establish engraftment in busulfan (BU) treated rats

Group	Treatment at Day		Days after grafting											
	−8	−1	0	7			14			21			28		
				Mort.	Chim.	GVHD	Mort.	Chim.	GVHD	Mort.	Chim.	GVHD	Mort.	Chim.	GVHD
1		BU	–	0/8	–	–	8/8	–	–	–	–	–	–	–	–
2		BU	Lewis	0/8	–	–	0/8	–	–	0/8	–	–	1/8	–	–
3		BU	ACI	0/8	–	–	8/8	–	–	–	–	–	–	–	–
4	RARTS	BU	ACI	1/8	0/7	0/7	2/8	6/6	4/6	4/8	4/4	4/4	7/8	1/1	1/1
5		BU+Cy	ACI	0/8	0/8	0/8	0/8	8/8	3/8	3/8	5/5	5/8	3/3	3/3	
6	CsA	BU	ACI	0/8	0/8	0/8	0/8	not tested	0/8	0/8	8/8	0/8	1/8	7/7	0/7

Groups of Lewis rats were treated with 30 mg/kg busulfan i.p. alone (groups 1, 2 and 3) or together with 150 mg/kg cyclophosphamide i.p. (group 5), a short course of rabbit anti-rat-thymocyte serum i.p. (RARTS, 1.5 ml/day daily from day −8 to day −2) (group 4) or cyclosporin A (25 mg/kg given subcutaneously daily from day −8 to day +18) (group 6). One day after busulfan administration 60×10^6 nucleated marrow cells were given intravenously. Cumulative mortality, fraction of animals proven to be chimeras and fraction of animals with clinical signs of GVHD are recorded. Chimerism was established by typing the peripheral lymphocytes with strain specific cytotoxic antisera

Table 2. Use of CsA to abolish sensitization to major histocompatibility antigens

Group	Days before transplant (sensitization)				Transplant	Days after transplant			
	Day −26[a]	Day −19	Day −18 thru Day −1	Day −1	Day 0	Day +1 thru +18	Day 7	Day 14	Day 21
1	–	–	–	Bu+Cy[b]	ACI marrow[c]	CsA daily	0/8[e]	0/8	0/8
2	–	ACI spleen	–	Bu+Cy	ACI marrow	–	0/8	8/8	–
3	–	ACI spleen	–	Bu+Cy	ACI marrow	CsA daily	0/8	8/8	–
4	–	ACI spleen	CsA daily[d]	Bu+Cy	ACI marrow	–	0/8	8/8	–
5	ACI spleen	ACI spleen	–	Bu+Cy	ACI marrow	–	0/8	8/8	–
6	ACI spleen	ACI spleen	CsA daily	Bu+Cy	ACI marrow	–	0/8	8/8	–
7	Lewis spleen	Lewis spleen	CsA daily	Bu+Cy	Lewis marrow	–	0/8	0/8	1/8

[a] Lewis rats were given 10^7 spleen cells from the prospective donor i.v. for sensitization
[b] They were then prepared with 30 mg/kg busulfan (Bu) and 150 mg/kg cyclophosphamide (Cy)
[c] 24 hours prior to receiving 60×10^6 nucleated marrow cells
[d] Cyclosporin A (CsA) was given daily for 18 days subcutaneously at a dose of 25 mg/kg
[e] Cumulative mortality is recorded

Table 3. Use of cyclosporin A to prevent GVHD in the MHC mismatched donor-recipient combination

Group No.	Treatment on day			Assessment on days[c]							
	−1	0[a]	1–18	14		21		28		84	
				Mort.	GVHD	Mort.	GVHD	Mort.	GVHD	Mort.	GVHD
1	1,000R	Lewis marrow	–	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
2	1,000R	Lewis marrow	CsA[b]	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
3	1,000R	ACI marrow	–	1/8	5/7	2/8	6/6	5/8	3/3	8/8	–
4	1,000R	ACI marrow	CsA	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8

Groups of Lewis rats were conditioned with 1,000 rads total body irradiation and grafted with syngeneic Lewis or allogeneic ACI marrow cells. CsA (25 mg/kg given subcutaneously daily from day −1 to +18) was administered to two groups. Cumulative mortality and fraction of animals with clinical signs of GVHD are recorded

Group No.	CsA post transplantation	GVHD days post transplantation			
		14	21	28	35
1	CsA days 1–+18	0/8	0/8	0/8	0/7
2	CsA days 3–+21	0/8	2/7	4/6	5/5
3	CsA days 7–+25	1/8	4/6	4/5	5/5
4	CsA days 14–+32	2/8	5/5	4/4	4/4
5	none	2/8	5/6	4/4	2/2

Groups of Lewis rats were conditioned with 1000 rads total body irradiation and grafted with allogeneic ACI marrow. CsA was given daily for 18 days at a dose of 25 mg/kg/day, starting at various times after transplantation. Fraction of animals with clinical signs of GVHD is recorded

Table 4. Use of CsA to treat GVHD. Recipients: Lewis; Donors: ACI; Conditioning 1000 rads

daily for 18 days starting at various times after grafting (days 1, 3, 7, 14). GVHD was prevented only, if CsA was given immediately after transplantation. A delay of only three days resulted in marked GVHD. Furthermore, CsA given at that dose was not capable of reversing an already established GVHD.

V. Use of CsA to Accelerate the Recovery of Immune Function After Allogeneic Marrow Transplantation

1. Functional and Histopathologic Studies

Groups of Lewis rats were given 1,000 rads TBI followed by 60×10^6 Lewis or ACI marrow cells. One group of rats was, in addition, given 25 mg/kg CsA s.c. daily for 18 days starting one day after marrow infusion. Four animals out of each group were sacrificed on day 28 and their lymphoid organs examined histologically. The relative number of lymphocytes was rated on a 0 to 4+ scale with 4+ being that of a normal Lewis rat. Furthermore, the spleen cells were subjected to mixed lymphocyte culture assays (see below).

The surviving animals were immunized with SRBC and the saline agglutination titers determined one week later. In addition, the animals were sensitized to DNCB and the delayed type hypersensitivity reaction read histologically. Again, the reaction was graded on a 0 to 4+ scale, 4+ being the reaction of a normal Lewis rat.

Table 5 summarizes the results of this experiment. Lewis rats grafted with histoincompatible marrow show marked lymphoid depletion of lymph nodes, spleen and thymus, and are unable to respond to SRBC or to demonstrate DTH to DNCB. Lewis rats grafted with syngeneic marrow display repopulation of the lymphoid organs as well as DTH and antibody response to SRBC. CsA treated allogeneic marrow recipients at 28 days after grafting are strikingly different from the allogeneic group not treated with CsA and show recovery of the immune status resembling that of the syngeneic control animals.

2. Proliferative Response of Allogeneic Lymphocytes (MLC)

Splenic lymphocytes from the above described recipients were harvested 28 days after transplantation and used as responders in a mixed lymphocyte culture

Table 5. Immune recovery of marrow transplant recipients after CsA treatment

Donor/recipient combination	ACI/Lewis[a] n=4	ACI/Lewis, CsA[b] n=4	Lewis/Lewis[c] n=4
Relative reconstitution of lymphoid tissue			
Thymus			
Cortex	all 1+	all 3+	3+,3+,4+,4+
Medulla	all 1	all 2+	all 4+
Spleen			
T-cell region	all 1+	all 3+	3+,3+,4+,4+
B-cell region	all 0	all 3+	all 4+
Lymph node			
T-cell region	all 1+	all 2+	3+,3+,4+,4+
B-cell region	all 0	all 2+	3+,3+,4+,4+
Delayed type hypersensitivity to DNCB	all 0	all 2+	all 3+
Antibody to SRBC (log_2)[d]	all 1	4,4,4,5	6,7,7,7

Groups of Lewis rats, (16 per allogeneic group, 8 per syngeneic group), were given 1000 rads TBI and injected with 60×10^6 nucleated ACI[a,b] or Lewis[c] marrow cells i.v. One group of rats[b] was, in addition, given 25 mg/kg CsA s.c. daily for 18 days starting one day after marrow infusion. Four animals out of each group were sacrificed at day 21 (and day 28 and day 35 in the CsA treated group) and the lymphoid organs examined histologically. The relative number of lymphocytes in the lymphoid tissues are rated on a 0 to 4+ scale with 4+ being that of a normal Lewis rat. Surviving animals were immunized i.v. with 1 ml of 10% solution of SRBC and the saline agglutination titers determined one week later[d]. In addition, the animals were sensitized to DNCB (600 μg/ml over 1 cm^2 of skin) on day 28, challenged on day 42 (60 μg/ml over 1 cm^2 of skin) and biopsied on day 44. The delayed type hypersensitivity reaction was graded on a 0–4+ scale, 4+ being the reaction of a normal Lewis rat

against host (Lewis) and third party (BN) lymphocytes. Splenic lymphocytes from allogeneic recipients not treated with CsA responded well to host and third party lymphocytes. Splenic lymphocytes from allogeneic recipients, however, treated with CsA, did not respond to host (Lewis) but to some extent to third party (BN) lymphocytes (Table 6).

3. *Suppressive Effect on the MLC of Splenic Lymphocytes from CsA Treated Chimeras*

Splenocytes from CsA treated chimeras were harvested 28 days after transplantation and added to mixed lymphocyte reactions between normal donor (ACI)

Responder	Stimulator	cpm ± SD
ACI	Lewis	18,712 ± 617
u-ch	Lewis	20,641 ± 1,311
u-ch	BN	13,774 ± 487
CsA-ch	Lewis	1,083 ± 101
CsA-ch	BN	4,663 ± 712

Table 6. Response of splenic lymphocytes from untreated ACI-Lewis chimeras (u-ch) and ACI-Lewis chimeras treated with CsA (CsA-ch) at 28 days after transplantation

Nature of additional cells	Number of additional cells	
	10×10^5	1×10^5
ACI, live		
(CPM ± SD)	23,912 ± 813	13,001 ± 915
(net CPM)[a]	+13,965	+2,287
Chimeric, live		
(CPM ± SD)	4,716 ± 908	11,643 ± 1,206
(net CPM)[a]	−5,231	+929
ACI, irradiated		
(CPM ± SD)	9,947 ± 912	10,714 ± 765
(net CPM)[a]	0	0
Chimeric, irradiated		
(CPM ± SD)	11,861 ± 1,329	10,419 ± 901
(net CPM)[a]	+1,914	−295

[a] Net CPM = CPM of responder + additional cells) vs stimulator] − [(responder + irradiated responder) vs stimulator]

Table 7. Addition of splenocytes from CsA treated chimeras or from normal ACI donors to an ACI-Lewis MLC (ACI responder − Lewis stimulator). Time of splenocyte harvest: 28 days post transplantation

responder and normal host (Lewis) stimulator cells (Table 7). Splenic lymphocytes from CsA treated chimeras suppressed the mixed lymphocyte reaction of donor type responder to host type stimulator cells, an effect which was dependent on the number of live, non-irradiated chimeric cells.

D. Discussion

The results described in this preliminary report indicate that the initial optimism for CsA generated by a number of earlier animal studies, appears to be justified: CsA when used in a strongly histoincompatible rat marrow transplant model was as immunosuppressive as CY and anti-thymocyte serum, permitting to establish a functioning permanent marrow graft across the major histocompatibility barrier. Clinically and histopathologically side effects were not noted, and no peri-transplantation mortality was observed, a finding quite in contrast to those obtained with other presently available immunosuppressive agents.

Most striking was the ability of CsA to completely prevent GVHD in a MHC-mismatched donor-recipient combination, when the drug was given prophylactically. GVHD remained absent even after CsA was discontinued, suggesting that immunological tolerance was rapidly established and maintained, a feature which again discriminates CsA from other presently available immunologically active compounds.

Corroborating these findings were the observed lymphoid repopulation kinetics, and the rather rapid development of immunological competence after transplantation, a process that in the allogeneic rat system used here generally takes more than 100 days to complete (Tutschka et al., 1973; Tutschka et al., 1979b).

It remains speculative how CsA accelerates the recovery of immune function post grafting that dramatically. It is possible that CsA acts directly as a stimulator

of the immune system. More likely is an indirect mechanism whereby CsA by preventing GVHD allows the immune system to regenerate more rapidly. This speculation is supported by animal data indicating that the degree and duration of the post-transplant immunoincompetence parallel the degree of histoincompatibility between donor and recipient and the degree, severity and duration of GVHD (Tutschka et al., 1973; Fass et al., 1973; Noel et al., 1978).

This powerful and unique new immunosuppressive and tolerance inducing agent was disappointing when used in two other problem areas of allogeneic marrow transplantation: It was incapable, even in combination with CY, at least at the chosen dose level, to overcome sensitization to major histocompatibility antigens and to prevent graft rejection, and secondly, it was not capable of reversing an already established, ongoing acute graft-versus-host reaction, a result similar to findings in another rat model (Borel et al., 1976a) and to very preliminary findings in human marrow transplantation (Powles et al., 1978). Both of these phenomena, host-versus-graft (rejection) and graft-versus-host reactions involve the efferent arm of cellular immunity and the activity of cytotoxic effector lymphocytes. The inability of CsA to effectively counteract the efferent arm of cellular immune reactions might indicate that CsA interferes with the antigen recognition phase rather than being inhibitory or cytotoxic to primed effector lymphocytes.

Studies by Leoni et al. (1978) indicated that CsA was toxic for human lymphoblasts of both T and B cell origin, but not for resting lymphocytes or cells of myeloid or other origins. This finding suggested that CsA might in vivo be capable of eliminating lymphocytes responding to specific antigens. White et al. (1979a), on the other hand, showed that CsA inhibited the proliferation of preferentially T-cells in antigen-independent and antigen-dependent proliferation assays, indicating that CsA inhibited cell proliferation rather than the antigen recognition phase.

Studies in our own laboratory revealed that CsA was cytotoxic to PHA induced blasts, but only if used in very high doses. Low doses of the drug inhibited lymphocyte proliferation to mitogens and drastically to allogeneic cells in primary responses. Secondary responses were inhibited but to a much lesser degree. Furthermore, lymphocytes, after removing CsA from the primary culture, could be restimulated with the same antigen (allogeneic lymphocytes), although the kinetics of the lymphocyte proliferation were different from a regular secondary MLC, suggesting that the effect of CsA was to some extent reversible (Hess et al., 1979).

This is in contrast to the behavior of lymphocytes from organ transplant recipients who were given CsA in vivo. Those lymphocytes showed specific donor hyporesponsiveness, and no evidence for the presence of suppressor cells could be demonstrated (White et al., 1979b).

Our studies reported here indicate that the specific donor-host tolerance established in the marrow grafted recipient was, at least in part, due to the rapid development of a suppressor cell system, capable of suppressing mixed lymphocyte reactions in vitro. Similar suppressor systems have been described by us in long term allogeneic rat bone marrow chimeras, suggesting that they represented the primary mechanism for establishing and maintaining transplan-

tation tolerance (Tutschka et al., 1977c; Tutschka et al., 1979a), a process that required several months to develop (Tutschka et al., 1979b).

Why CsA is capable of operationally inducing suppressor cells that rapidly is presently unclear. It is possible that CsA directly stimulates a subset of lymphocytes with suppressor capacities, more likely appears a process whereby CsA by preventing proliferative and cytolytic responses to transplantation antigens permits indirectly the development of suppressor cells, tipping the balance towards transplantation tolerance and a stable chimeric state.

Thus, CsA is not only a promising agent of a new family of immunosuppressive drugs with tremendous clinical potential, it might also become a new investigative tool to unravel the complex immunologic processes associated with graft-versus-host reactions and transplantation tolerance.

Acknowledgements

We acknowledge the excellent technical assistance of Mrs. Lynn Smith and the excellent secretarial help of Mrs. Joan Ziolkowski. We appreciate the helpful discussions with Dr. George W. Santos and Dr. Anthony C. Allison. We thank Drs. Wiskott and Lardi from Sandoz, Inc. (Basel, Switzerland) for generously supplying us with CsA.

References

1. Borel, J. F., Feurer, C., Gubler, H. U., Stähelin, H.: Biological effects of cyclosporin A: A new antilymphocytic agent. Agents Actions *6,* 468–475 (1976a)
2. Borel, J. F.: Comparative study of in vitro and in vivo drug effects on cell-mediated cytotoxicity. Immunology *31,* 631–641 (1976b)
3. Borel, J. F., Feurer, C., Magnée, C., Stähelin, H.: Effects of the new anti-lymphocytic peptide cyclosporin A in animals. Immunology *32,* 1017–1025 (1977)
4. Calne, R. Y., White, D. J. C., Rolles, K., Smith, D. P., Herbertson, B. M.: Prolonged survival of pig orthotopic heart grafts treated with cyclosporin A. Lancet *1978 I,* 1183–1185 (1978)
5. Elfenbein, G. J., Anderson, P. N., Humphrey, R. L., Mullins, G. M., Sensenbrenner, L. L., Wands, J. R., Santos, G. W.: Immune system reconstitution following allogeneic bone marrow transplantation in man: A multi-parameter analysis. Transplant. Proc. *8,* 641–646 (1976)
6. Elfenbein, G. J., Anderson, P. N., Klein, D. L., Schacter B. Z., Santos, G. W.: Difficulties predicting bone marrow graft rejection in patients with aplastic anemia. Transplant. Proc. 2/10, 441–445 (1978)
7. Fass, L., Ochs, H. D., Thomas, E. D., Mickelson, E., Storb, R., Fefer, A.: Studies of immunological reactivity following allogeneic marrow grafts. Transplantation *16,* 630–640 (1973)
8. Gale, R. P., Opelz, C., Mickey, M. R., Graze, P. R., Saxon, A. for the UCLA Bone Marrow Transplant Teamt: Immunodeficiency following allogeneic bone marrow transplantation. Transplant. Proc. *10,* 223–227 (1978)
9. Grebe, S. C., Streilein, J. W.: Graft-versus-host disease. Adv. Immunology *22,* (1976)
10. Green, C. L., Allison, A. C.: Extensive prolongation of rabbit kidney allograft survival after short-term cyclosporin A treatment. Lancet *1978 I,* 1182–1183
11. Hess, A. D., Matyas, J. R., Tutschka, P. J.: Inhibition of in vitro human lymphocyte responses by cyclosporin A. Experimental Hematology, submitted (1979)
12. Humphrey, R. L., Santos, G. W.: Serum protein allotype markers in certain inbred rat strains. Fed. Proc. *20,* 314 (1971)
13. Khouri, N., Saral, R., Armstrong, E. M., Tutschka, P. J., Santos, G. W., Beschorner, W. E.,

Siegelman, S. S.: Pulmonary interstitial changes following bone marrow transplantation. Radiology, (in press)
14. Leoni, P., Garcia, R. C., Allison, A. C.: Effects of cyclosporin A in vitro. J. Clin. Lab. Immunol. *1*, 67 (1978)
15. Lichter, A. Tutschka, P. J., Wharam, M., Elfenbein, G. J., Sensenbrenner, L. L., Saral, R., Kaizer, H., Santos, G. W., Order, S. E.: The use of fractionated radiotherapy as preparation for allogeneic bone marrow transplantation. Transplant. Proc. *11*, 1492–1494 (1979)
16. Neiman, P. E., Reeves, W., Ray, G., Flourney, N., Lerner, K. G., Sale, G. E., Thomas, E. D.: Prospective analysis of interstitial pneumonia and opportunistic viral infection among recipients of allogeneic marrow grafts. J. Infect. Dis. *136*, 754–767 (1977)
17. Noel, D. R., Witherspoon, R. P., Storb, R., Atkinson, K., Doney, K., Mickelson, E. M., Ochs, H. D., Warren, L. P., Weiden, P. L., Thomas, E. D.: Does graft-versus-host disease influence the tempo of immunologic recovery alter allogeneic human marrow transplantation? An observation on 56 long-term survivors. Blood *51*, 1087–1105 (1978)
18. Petcher, T. J., Weber, H. P., Rüegger, A.: Crystal and molecular structure of an Iodo-derivative of the cyclic undecapeptide cyclosporin A. Helv. Chim. Acta *59*, 1480–1489 (1976)
19. Powles, R. L., Barrett, A. Z., Clink, H., Kay, H. E. M., Sloane, J., Mc Elwain, T. J.: Cyclosporin A for the treatment of graft-versus-host disease in man. Lancet *1978 II*, 1327–1331
20. Rüegger, A., Kuhn, M., Lichti, H., Loosli, H.-R., Huguenin, R., Quiquerez, C., Wartburg, A. von: Cyclosporin A, ein immunsuppressiv wirksamer Peptidmetabolit aus Trichoderma polysporium (Link ex Pers) Rifai. Helv. Chim. Acta *59*, 1075–1092 (1976)
21. Santos, G. W., Owens, A. H., Jr.: A comparison of the effects of selected cytotoxic agents on allogeneic skin graft survival in rats. Bull. Hopkins Hosp. *116*, 327–329 (1965)
22. Santos, G. W., Owens, A. H., Jr.: 19s and 7s antibody production in the cyclophosphamide or methotrexate treated rats. Nature *209*, 622–624 (1966)
23. Santos, G. W., Sensenbrenner, L. L., Anderson, P. N., Burke, P. J., Klein, D. L., Slavin, R. E., Schacter, B., Borgaonkar, D. S.: HL-A identical marrow transplants in aplastic anemia, acute leukemia, and lymphosarcoma employing cyclophosphamide. Transplant. Proc. *8*, 607–610 (1976)
24. Santos, G. W.: Bone marrow transplantation. In: Advances in internal medicine, Vol. *24*, pp. 157–182. Chicago: Year Book Medical Publishers 1979
25. Slavin, R. E., Tutschka, P. J., Santos, G. W.: Contact sensitization to 2–4 Dinitrochlorobenzene (DNCB) in normal, cyclophosphamide (CY) treated and marrow grafted rats. Fed. Proc. *32*, 879 (1973)
26. Storb, R., Epstein, R. B., Rudolph, R. H., Thomas, E. D.: The effect of prior transfusion on marrow grafts between histocompatible canine siblings. J. Immunol. *105*, 627–633 (1970)
27. Storb, R., Thomas, E. D., Weiden, P. L., Buckner, C. D., Clift, R. A., Fefer, A., Goodell, B. W., Johnson, F. L., Neiman, P. E., Sanders, J. E., Singer, J.: One hundred ten patients with aplastic anemia (AA) treated by marrow transplantation in Seattle. Transplant. Proc. *10*, 135–140 (1978)
28. Thomas, E. D., Storb, R., Clift, R. A., Fefer, A., Johnson, F. L., Neiman, P. E., Lerner, K. G., Glucksberg, H., Buckner, C. D.: Bone marrow transplantation. N. Engl. J. Med. *292*, 832–843 and 895–902 (1975)
29. Tutschka, P. J., Slavin, R. E., Santos, G. W.: Immunological studies in bone marrow grafted rats. Exp. Hematol. *1*, 287 (1973)
30. Tutschka, P. J., Santos, G. W.: Marrow transplantation in the Busulfan treated rat. I. Effect of cyclophosphamide and rabbit-anti-rat thymocyte serum as immunosuppression. Transplantation *20*, 101–106 (1975a)
31. Tutschka, P. J., Santos, G. W.: Bone marrow transplantation in the Busulfan treated rat. II. Effect of cyclophosphamide and anti-thymic serum on the presensitized state. Transplantation *20*, 116–122 (1975b)
32. Tutschka, P. J., Saral, R., Elfenbein, G. J., Sensenbrenner, L. L., Bias, W., Borgaonkar, D., Slavin, R. E., Santos, G. W.: Marrow transplant – aplastic anemia. Summary of Baltimore experience. Exp. Hematol. *5*, 46 (1977a)
33. Tutschka, P. J., Santos, G. W.: Bone marrow transplantation in the Busulfan treated rat. III. Relationship between myelosuppression and immunosuppression for conditioning bone marrow recipients. Transplantation *24*, 52–62 (1977b)
34. Tutschka, P. J., Schwerdtfeger, R., Slavin, R., Santos, G. W.: Mechanism of donor to host

tolerance in rat bone marrow chimeras. In: Experimental hematology today. Baum, S. J., Ledney, G. D. (eds.), pp. 191–197. New York: Springer, 1977b
35. Tutschka, P. J., Santos, G. W., Sensenbrenner, L. L., Elfenbein, G. J., Saral, R., Kaizer, H., Lichter, A., Wharam, M., Order, S. E.: Allogeneic bone marrow transplantation (BMT) as therapy for acute leukemia – Summary of Baltimore experience. Exp. Hematol. *6*, 7 (1978)
36. Tutschka, P. J., Santos, G. W., Beschorner, W.: The role of suppressor cells in transplantation tolerance. Transplant. Proc. *11*, 882–886 (1979a)
37. Tutschka, P. J., Hutchins, G. W., Santos, G. W.: Suppressor cells as possible mediators of immune function after marrow transplantation. In: Experimental hematology today. Baum, S. J., Ledney, G. D. (eds.), pp. 113–121. New York: Springer, 1979b
38. Tutschka, P. J.: Allogene Knochenmarktransplantation als Therapie für Aplastische Anämie und Akute Leukämie. In: Immunforschung für Klinik und Labor. Thierfelder, S. (ed.), pp. 107–122. Friedberg: Bindernagel, 1979c
39. Weiss, A., Fitch, F. W.: Macrophages suppress CTL generation in rat mixed leukocyte cultures. J. Immunol. *119*, 510–516 (1977)
40. White, D. J. G., Plumb, A. M., Pawelec, G., Brons, G.: Cyclosporin A: An immunosuppressive agent preferentially against proliferative T cells. Transplantation *27*, 55–58 (1979a)
41. White, D. J. G., Calne, R. J., Herbertson, B. M., Plumb, A.: Mode of action of cyclosporin A: A new immunosuppressive agent. Transplant. Proc. *11*, 855 (1979b)

Discussion

Ivanyi: Did you try spleen cells for GVH with cyclosporin A?
Tutschka: No, we are doing it presently. I would not expect an effect on sensitized spleen cells because cyclosporin A has in our hands not shown a very good effect against secondary responses.
Thierfelder: Do you think that cyclosporin A is a T cell agent?
Tutschka: We do not know what the mechanism of action of cyclosporin A is. Two possibilities among others can be discussed: first, there is a cytotoxic effect to blasts against alloantigens or PHA at high doses. On the other hand we have shown with rather low doses in MLC that we were able to induce suppressor cells. Rats surviving 250 days after transplantation and recovering from graft-versus-host disease have very specific suppressor cells. It looks as if cyclosporin reduces the time necessary for the establishment of tolerance by suppressor cells to about 20 days.
Simonsen: Did you test for chimerism?
Tutschka: With cytotoxic antisera the thymocytes and spleen cells were all donor type.

Cyclosporin A in Human Bone Marrow Grafts

H. E. M. Kay, R. L. Powles, J. P. Sloane, and M. G. Farthing

Cyclosporin A has been used to treat graft versus host reactions in eight patients of whom seven had had marrow grafts for acute leukemia, and one for marrow aplasia. All grafts were MHC-MLR compatible. The drug, in doses up to 2100 mg daily, has been given either by intramuscular injection, or by mouth in capsules or dissolved in olive oil.

Details of the eight cases are shown in Tables 1 and 2 (cases 1–5 have been the subject of a preliminary report) [1].

The first three patients all had evidence of moderate or severe GVH affecting the liver and bowel as well as the skin. Treatment with CsA began as soon as the skin biopsy showed GVH of grade II or more. In all three there was rapid response of the skin condition with fading erythema, but diarrhoea and hepatic function deteriorated and they died with jaundice, oedema and ascites.

At autopsy in cases 1 and 2 the liver showed cholestasis with bile duct atypia and veno-occlusive disease associated with centrilobular congestion and loss of

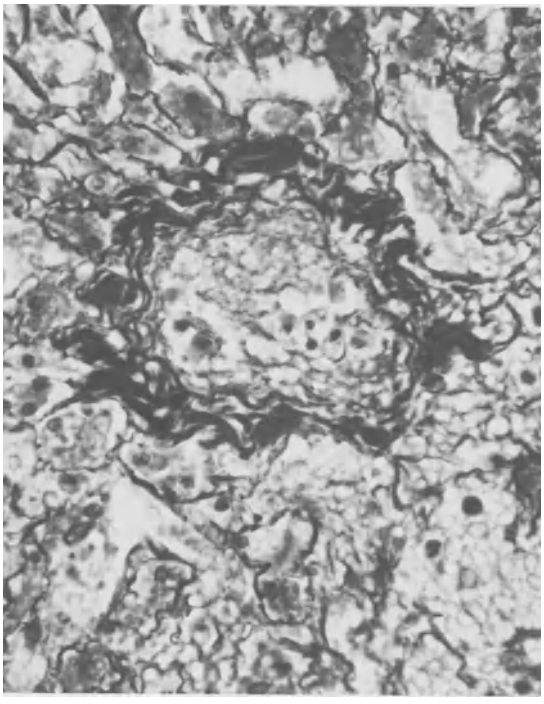


Fig. 1. Case 1. Centrilobular vein showing occlusion by a network of reticulin with a few cells (Reticulin × 400)

Table 1. Five patients receiving cyclosporin A for GVH

Case	Age	Sex		Condition	Graft-GVH (days)	Skin	Liver	Bowel	Viruses
		Donor	Recip.						
1	14	F	M	AMML 1st rem	17	Grade II resolved in 6 days	Cholestasis Bile duct atypia Centrilobular fibrosis	Progressive diarrhoea	0
2	21	F	M	AML 2nd rem	26	Grade II clinical improvement	Cholestasis Bile duct atypia Centrilobular fibrosis	Progressive diarrhoea	0
3	33	F	F	AML 1st rem	17	Grade III-IV resolved in 6 days	Slight focal necrosis	Progressive diarrhoea	0
4	35	M	M	AML 3rd relapse	13	Grade III resolved in 9 days	Some cholestasis and necrosis with CMV inclusions	No symptoms Jejunum normal	ECHO 11 Zoster CMV
5	17	F	M	AUL 1st rem	23	Acute grade III later chronic with slow improvement	Moderate enzyme elevations	Normal	ECHO 11 Zoster CMV

Table 2. Three cases where GVH was first diagnosed after stopping prophylactic cyclosporin A

Case	Age	Sex		Condition	Prophylactic CSA	Skin	Liver	Bowel
		Donor	Recip.					
6	20	M	M	Marrow aplasia	D−1 to D+16	Grade II D+29	Jaundice from D+12 cholestasis Bile duct atypia	Progressive diarrhoea from D+20 rectum grade III D+29
7	34	F	M	AML 1st rem	D−1 to D+42	Grade III resolved D+48	Enzymes↑ biopsy normal	Rectal biopsy grade II D+48
8	19	M	M	ALL 2nd rem	D−1 to D+31	Grade III D+32	Enzymes↑ biopsy normal	Rectal biopsies normal

AMML – Acute myelomonolytic leucemia, AML – Acute myeloid leucemia, AUL – Acute undifferentiated leucemia, ALL – Acute lymphoblastic leucemia, CMV – Cytomegalovirus

hepatocytes (Fig. 1) but in case 3 the liver showed only small foci of necrosis and siderosis. All had severe ulceration and loss of intestinal mucosa.

Case 4 had only mild evidence of hepatic dysfunction and a jejunal biopsy showed no abnormality. An Echo 11 virus had been isolated from his pharynx (a virus that happened to be very common in England at the time) and later he developed a vesicular rash from which zoster virus was isolated and revealed by E. M. The rash was never very extensive or hemorrhagic but he had two generalised convulsions with fatal cardiac arrest after the second. Autopsy showed viral inclusions in the liver but no zoster or other virus in the brain and/or cardiac abnormality to account for cardiac arrest. Thus the immediate cause of death is uncertain and it is not clear whether cyclosporin A, added to the hazard of the virus infection, may have been in part responsible for this unexpected death.

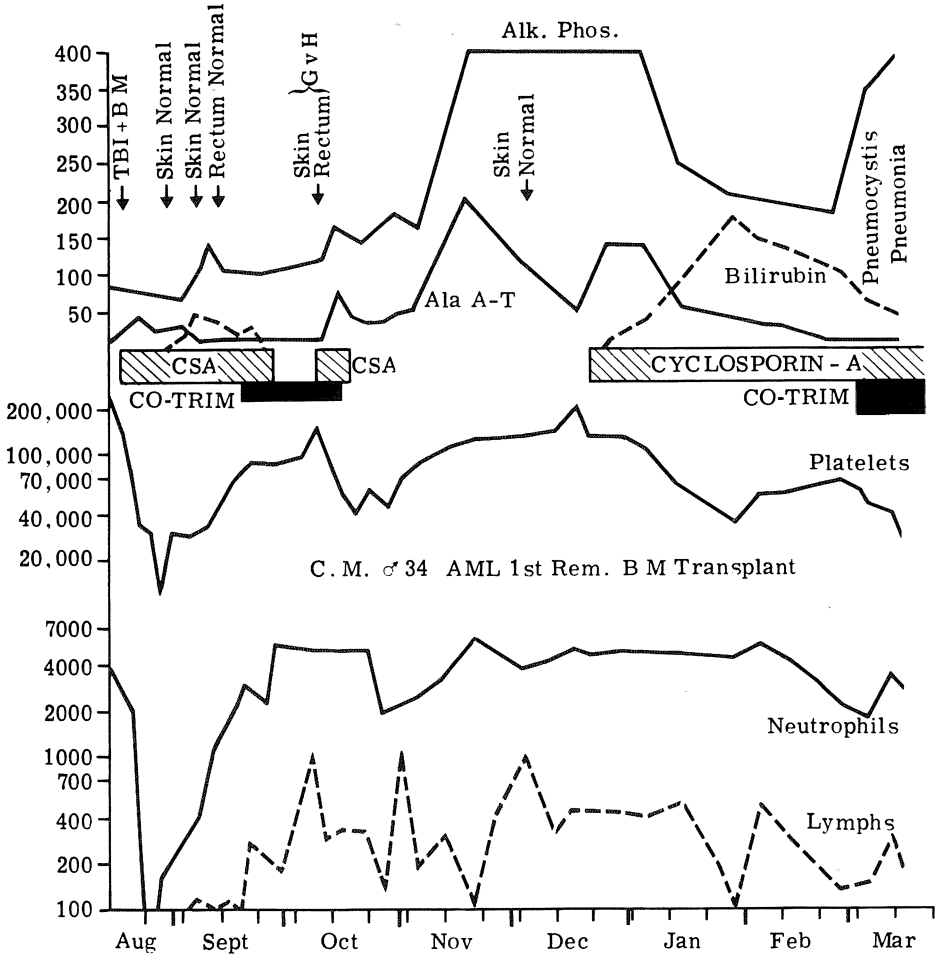


Fig. 2. Case 7. Liver function and blood counts in relation to cyclosporin A (CsA) administration. Co-trim = Co-trimoxazole; Ala A-T = Alanine amino-transferase

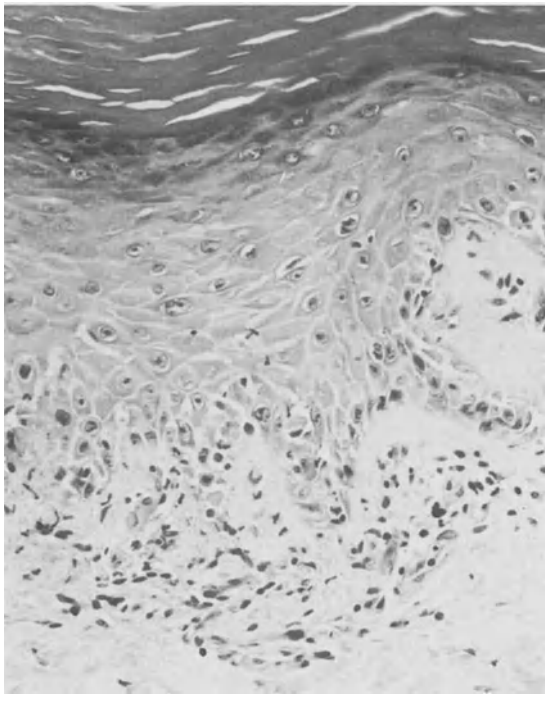


Fig. 3. Skin biopsy case 7 showing grade III GVH on day 49 (H and E×250)

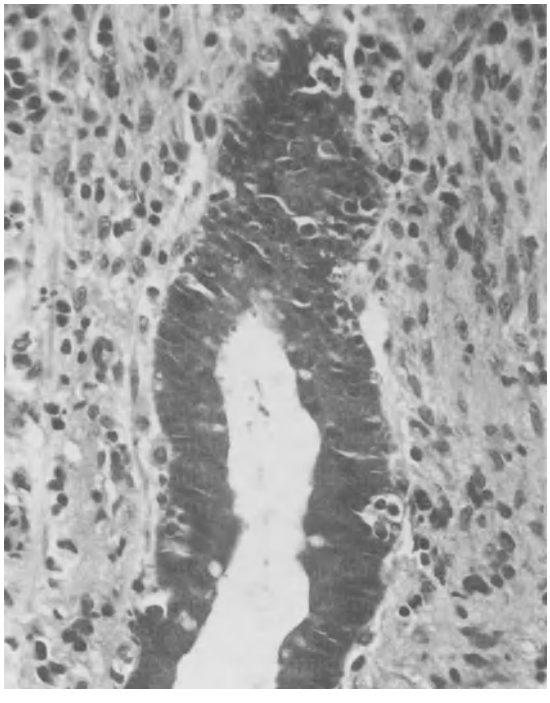


Fig. 4. Rectal biopsy case 7 grade III GVH on day 49 (H and E×250)

Case 5 has had only slight evidence of hepatic dysfunction and none of bowel disease. He survives with chronic graft-versus-host skin disease which is now showing some improvement. The skin is deeply pigmented but its texture is improved and he has a full range of painless movement in all areas except the neck where a severe attack of zoster on the left side is now healing.

Table 2 refers to three patients where cyclosporin A was given from the day before transplantation as prophylaxis. All three had transient and non-specific erythematous rashes while on CsA but the biopsies at this time showed neither the lymphocytic infiltration nor the cell necrosis needed to establish the presence of active GVH.

CsA was discontinued electively in cases 7 and 8 but in case 6 the main reason was renal and hepatic dysfunction. This patient developed severe GVH. The skin responded to CsA but the liver and intestinal disease continued unabated. ATG was also without effect.

The course of events in case 7 is shown in Fig. 2. Skin and rectal biopsies first became positive soon after discontinuing CsA six weeks from the day of transplant (Figs. 3, 4). He has mild chronic GVH affecting skin, liver and intestine. Cyclosporin A appears to reduce the skin erythema and itching, does not obviously affect the bowel condition and may or may not be responsible for the fluctuations in his hepatic function. Liver biopsy shows only some siderosis.

In case 8 the skin biopsy showed grade III changes the day after CsA was topped which must be taken as evidence that even skin GVH is not always suppressed by CsA.

Most of the patients have had evidence of moderate or severe renal impairment while on CsA with blood urea rising to over 20 mmol/l and creatinine clearances declining to under 10 ml/minute in two cases. These effects are fairly quickly reversed on stopping the drug and in patients who come to autopsy there has been no histological evidence of renal damage.

In summary cyclosporin A, which in experimental studies has had a strong immunosuppressive action, is of only moderate value in GVH in man. Skin GVH does respond both symptomatically and as shown by biopsy but there is no evidence that liver of bowel GVH is amenable to treatment and no conclusion can yet be reached on the value of cyclosporin A as prophylaxis.

Acknowledgements

These patients were cared for in the Leukaemia Unit of the Royal Marsden Hospital. We are indebted to Dr. J. F. Borel of Sandoz Limited for the provision of cyclosporin A and to the Bud Flanagan Leukaemia Fund and the Leukaemia Research Fund for support.

Reference

1. Powles, R. L., Barrett, A. J., Clink, H., Kay, H. E. M., Sloane, J., McElwain, T. J.: Cyclosporin A for the treatment of graft versus host disease in man. Lancet *1978 II,* 1327–1331

Discussion

Storb: We are doing a study with cyclosporin in histo-incompatible dogs. Basically we are seeing the same thing that you are seeing. You can attenuate graft-versus-host disease as long as you give the drug. When you discontinue it the dogs develop GVH disease. When you give it early on after transplantation, the survival infact was worse and we have seen a fair amount of gastrointestinal toxicity and lost those animals earlier than their control counterparts. We also found that one can reverse skingraft rejection with the drug and that dog's lymphocytes that are being treated with cyclosporin respond normally with the skin graft donors' lymphocytes in mixed lymphocyte culture and CML reactions in pooled normal serum whereas if you add the dog's serum back to the lymphocytes you attenuate everything. It seems to be an agent that has a reversible effect on lymphocytes. It is not lymphocytotoxic in our hands. We failed to induce tolerance, so the dog behaves different from the rat.

Santos: May I ask Dr. Storb and Dr. Kay, was the prophylactic treatment immediately after transplantation without the addition of any other agents because, quite clearly, in our hands with the addition of any other agent the toxicity is enhanced as Dr. Tutschka illustrated. Were your patients treated simply with cyclosporin A?

Kay: That's right, from day 0, the moment they finished radiation

Storb: No, because the dogs did not take anything p.o.

6 Autologous Bone Marrow Transplantation

Immunological Studies in Patients Submitted to Autologous Bone Marrow Transplantation

A Preliminary Report*

N. C. Gorin, J. Y. Muller, C. Salmon, and G. Duhamel

A. Summary

The immune competence of 12 patients who received a high dose combination chemotherapy regimen (TACC), was assayed before chemotherapy and for periods of up to 200 days after chemotherapy. Of these 12 patients, 9 were grafted with cryopreserved autologous marrow and 3 were from a group of 5 patients who were not grafted.

Following transplant, circulating immunoglobulin levels fell moderately and remained depressed less than 2 months for IgG, and for variable and longer periods of time for IgM and IgA. Repeated quantitative determinations of antibodies against multiple viral antigens and bacterial antigens did not show any decrease in the pretransplant levels. Indeed some patients developed herpes and cytomegalovirus infections to which they responded by a sharp increase in antibody titers. In 2 cases, a primary immunization was demonstrated (against CMV and BK virus) with increasing levels of IgM and IgG antibodies.

Lymphocyte counts in peripheral blood returned to 500 mm^{-3} between day 10 and 29 (median day 18) and to pretransplant values within 6 weeks. Non specific stimulation of lymphocytes by mitogens in the immediate post-transplant period showed a decreased response to PHA and Con A, whereas the responses to pokeweed mitogens and alloantigens were only slightly diminished. The degree of the responses was related to the dose of cryopreserved marrow infused.

The results of this study, compared to the results of similar studies reported by others after allogeneic bone marrow transplantation, suggest the following conclusions:

1. Although the minimum dose for autologous bone marrow transplantation in man is around 0.5 10^8 nucleated bone marrow cells/kg, much higher doses should be used to ensure faster and better restoration of immune reactivity.
2. The similarity of the immunological dysfunction following autologous and allogeneous bone marrow transplantation suggest that, in the immediate post-transplant period, the role of GVHD in cellular immunity depression may be minimal.

B. Introduction

From January 1976 to January 1979, 14 patients admitted to our department were submitted to a high dose combination chemotherapy regimen (TACC). Ten

* Supported by a grant from DGRST No. 77 7 1374

of these patients received autologous cryopreserved marrow after completion of chemotherapy and four did not. The immune competence of 12 of these patients has been periodically evaluated. This study has been carried out in an effort to characterize autologous immune reconstitution, compare it to allogeneic immune reconstitution and from this comparison hopefully get a better understanding of the responsibility of graft versus host disease (GVHD) in the depression of immunity. The results of this study are presented here, at this international seminar, as a preliminary report.

C. Material and Methods

1. Patients

Ten patients received high dose combination chemotherapy (TACC) and autologous cryopreserved marrow. 6 of these patients had solid tumors without bone marrow involvement and 4 patients had drug resistant acute leukemia in relapse. All patients recovered a WBC count of 1000/mm3 within 19 days post-infusion of cryopreserved marrow and were considered as successfully grafted.

9 of these patients were evaluated for immune status, for periods up to 200 days post-autologous bone marrow transplantation.

4 patients received the same high dose combination chemotherapy regimen, but no cryopreserved marrow. All 4 patients had drug resistant acute leukemia. One of these patients died too early to be evaluated. For the 3 other patients, the survival was much shorter than in the group of patients receiving cryopreserved marrow and therefore, the period of study did not exceed 80 days.

2. Bone Marrow Harvesting, Freezing and Storage

The technique for bone marrow harvesting, freezing and storage has been extensively described elsewhere (Gorin et al., 1979) and is similar to the technique we have previously used in dogs and which resulted in a 100% stem cell preservation (Gorin et al., 1978a, c).

3. High Dose Combination Chemotherapy (TACC)

The basic 4 days course of TACC consisted of:
Cyclophosphamide 45 mg/kg IV day 1–4
ARA-C 100 mg/m2 IV every 12 hr day 1–4
6 Thioguanine 100 mg/m2 by mouth every 12 hr day 1–4
CCNU 200 mg/m2 by mouth day 2.

4. Autologous Engraftment

Frozen marrow was thawed rapidly in a water bath at 37° C and infused immediately, with no attempt to remove DMSO or destroyed red cells. The marrow cells were then administered by intravenous infusion without a filter, 48 h after the last dose of cyclophosphamide, and at least 72 h after the CCNU. The bags of bone marrow were administered 2 at a time to avoid renal damage from haemoglobinuria. Forced diuresis (4 l/m2) initially started with the administration of cyclophosphamide, was continued until urine cleared of hemoglobin. Dexchlorpheniramine maleate was given to counteract the possible effects of histamine release associated with IV DMSO.

Except for the first patient, all patients were treated in a protected environment and received oral non-absorbable antibiotics for bowel decontamination.

5. Results of Chemotherapy and Autologous Bone Marrow Transplantation (Table 1)

Table 1 summarizes the dose of bone marrow infused, the duration of storage, the kinetics of recovery of hemopoiesis, the tumor cytoreduction and the survival of all patients. Details of the kinetics of recovery of hemopoiesis have been published elsewhere (Gorin et al., 1979).

Table 1. Recovery of peripheral blood cells, tumor response and survival in patients treated with high dose combination chemotherapy (TACC) with and without cryopreserved autologous marrow (14 patients)

Diagnosis	Dose of marrow (10^8/Kg)	Storage duration (months)	Leukocytes >1,000/mm^3 (day)	Time for Recovery[a]			Number of platelet[b] transfusions	Tumor response and Duration of response (days)	Survival (days)
				PMN >500/mm^3 (day)	Platelets >50,000/mm^3 (day)	Reticulocytes >0.1% (day)			
Nasopharyngeal carcinoma	2.2	1	17	17	15	16	2	PR	120
Rhabdomyosarcoma	0.8	0.5	12	13	15	13	5	PR	132
Hodgkin's disease stage IV	1.9	1.5	18	17	20	21	5	CR 100	360+
Hodgkin's disease Stage IV	1	0.5	13	13	17	16	5	CR 45	123
Diffuse histiocytic[d] lymphoma	1.6	0.5	10	20	45	14	18	CR 300+	300+
Localized plasmacytoma	0.6	5	16	13	9	8	1	PR	330+
A.M.L.	0.5	6	17	18	23	18	8	CR 83	105
A.M.L.	1	18	19	22	14	15	5	CR 280+	280+
A monoblastic L	1.4	3	19	19	28	25	9	CR 145	152
A monoblastic L	0.6	3.5	14	17	14	12	13[e]	CR 90	136
A.L.L.			30	29	33	27	18	CR 30	64
A.M.L.			28 (9% blast cells)	28	NO	NO	–	Failure	56
CML in acute crisis	No infusion of autologous marrow		27 (77% blast cells)	NO	NO	26	–	Failure	90
CML in acute crisis				†on day	15				

[a] Columns titled leukocytes, PMN, platelets and reticulocytes represent the time for these to return to the stated level. Day O is the day of marrow infusion or the corresponding day for patients who did not receive cryopreserved autologous marrow

[b] Number of platelet transfusions to maintain the platelet count above 30,000/mm^3

[c] CR = complete remission, PR = partial remission

[d] This patient received 2 consecutive doses of CCNU and cryopreserved marrow 48 hours only after the last dose, which probably accounts for the long lasting thrombocytopenia.

[e] Patient strongly immunized

D. Results

1. Lymphocyte Counts

In the group of patients receiving cryopreserved marrow, the lymphocyte count in peripheral blood returned to 500/mm3 on days 10–28 (median day 18), and to 1000/m3 on days 11–100 (median day 37). The estimated recovery of circulating lymphocytes, a measurement which takes into account the lymphocyte counts before high dose chemotherapy and ABMT, showed a 100% recovery in 8 patients, a 60 to 75% recovery in one patient with Hodgkin's disease, and a 70 to 100% recovery in one patient with AML.

2. Immunoglobulin Determinations (Figs. 1–3)

The concentrations of specific immunoglobulin fractions generally remained within normal limits during the post-transplant period.

Plotting the results for each individual patient however, showed that following chemotherapy and transplant, circulating immunoglobulin levels fell moderately for all patients but one, patient G, who for some reason had an increase in IgA and IgM levels in the immediate post-transplant period. This patient received the highest dose of cryopreserved marrow (2.2 10^8 nucleated cells/kg).

IgG globulins remained depressed for only a very short period of time and returned to pretransplant levels within 30 to 70 days (Fig. 1). On the contrary,

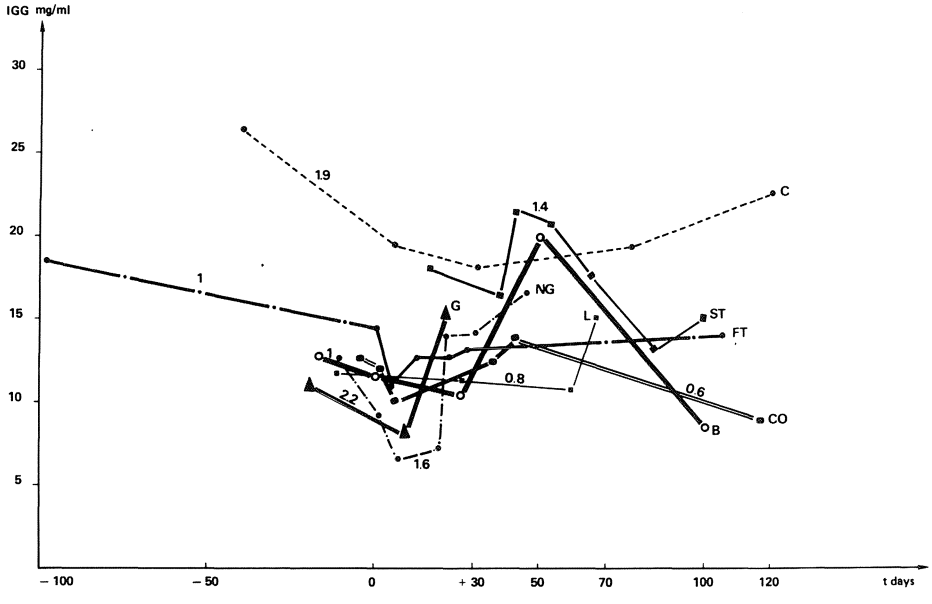


Fig. 1. IgG levels following TACC and autologous bone marrow transplantation (The numbers above each curve indicate the doses of bone marrow infused: $\times 10^8$ nucleated cells/kg)

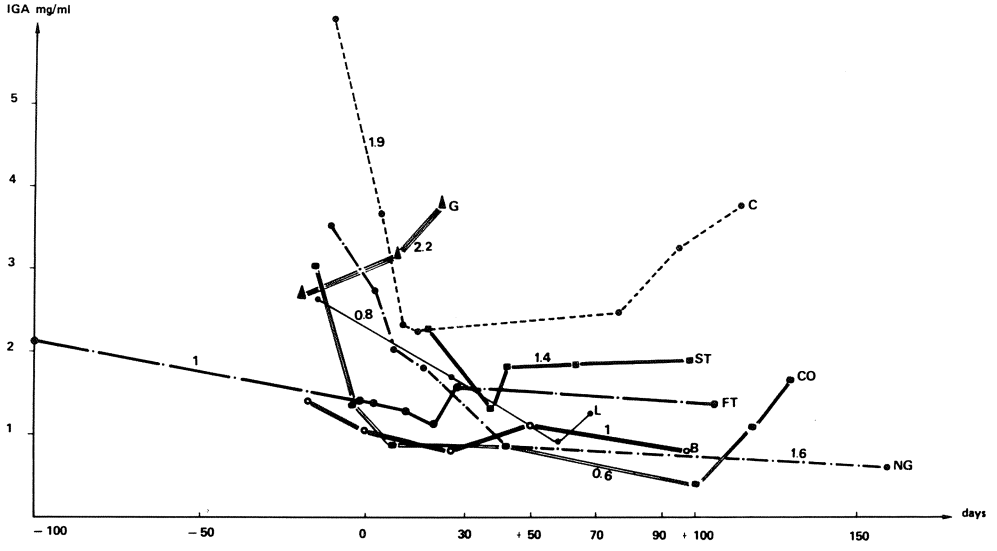


Fig. 2. IgA levels following TACC and autologous bone marrow transplantation (The numbers indicate the doses of bone marrow infused: $\times 10^8$ nucleated cells/kg)

IgA and principally IgM remained depressed for longer and more variable periods of time (Figs. 2–3).

Patients receiving the higher doses of cryopreserved marrow seemed to have a faster and more complete recovery of pretransplant immunoglobulin levels, except for patient NG who received radiotherapy (25 Grays) to the mesenteric root, beginning 90 days post-transplant.

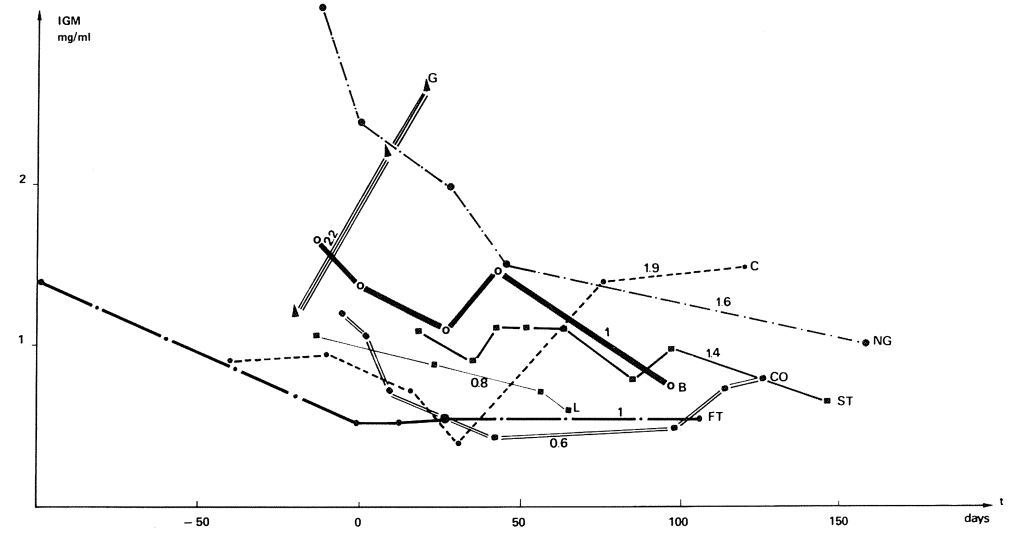


Fig. 3. IgM levels following TACC and autologous bone marrow transplantation (The numbers indicate the doses of bone marrow infused: $\times 10^8$ nucleated cells/kg)

A homogeneous component of immunoglobulins was not detected in any case.

3. Antibody Titers

Repeated quantitative determinations of antibodies against multiple viral and bacterial antigens, did not show any decrease from pretransplant levels. A seroconversion (2 dilution rise in the titer) was observed in 3 cases against cytomegalovirus, 4 cases against herpes virus, 1 case against varicella zoster virus and 1 case against BK virus (papova virus). In addition, the presence of IgM antibodies was detected in 2 instances (anti CMV and anti BK virus antibodies) demonstrating primo-immunization.

3 patients showed clinical evidence of localized herpes virus infection. 2 of these patients responded by increasing their antibody titers, while one failed to do so. A single case of interstitial pneumonitis occured following ABMT and was possibly related to pneumocystis carinii infection. It rapidly resolved following trimethoprim-sulfamethoxazole therapy.

4. In Vitro Lymphocyte Response (Figs. 4–7)

In vitro lymphocyte response was not evaluated for every patient at each time interval. The results however, showed a significantly diminished response to PHA and Con A up to 200 days post transplant. In contrast, the responses to pokeweed mitogen and to alloantigens in mixed lymphocyte cultures were only slightly

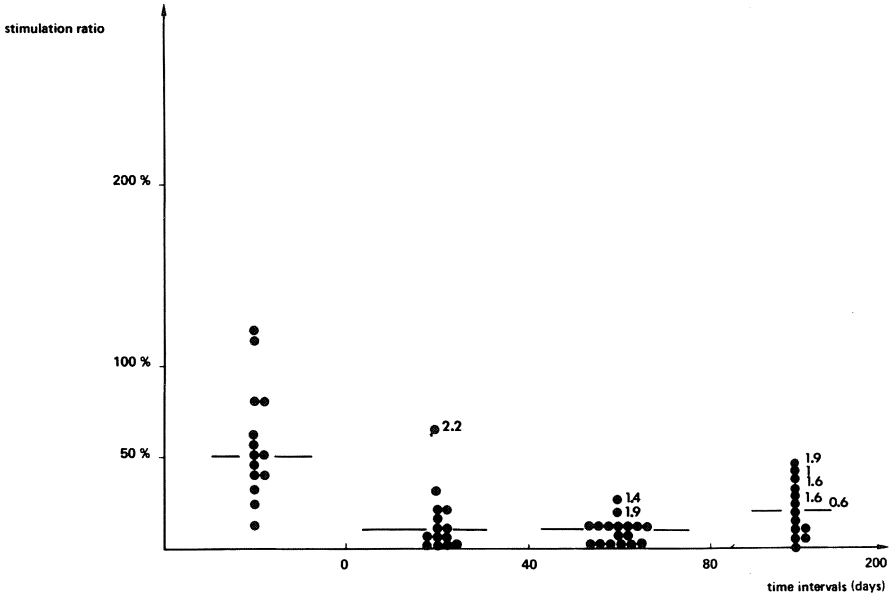


Fig. 4. In vitro lymphocyte response to PHA following autologous bone marrow transplantation (The numbers indicate the doses of nucleated bone marrow cells infused: $\times 10^8$/kg)

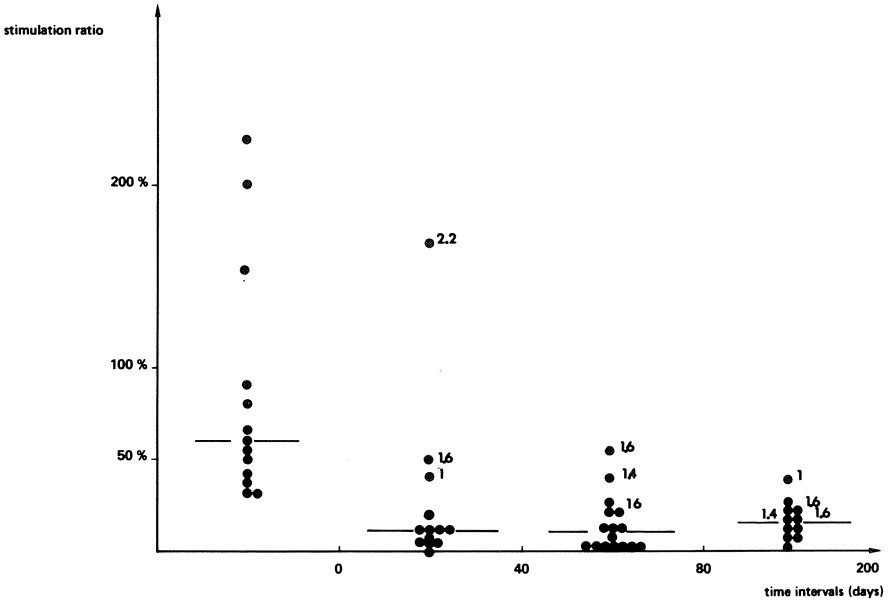


Fig. 5. In vitro lymphocyte response to Concavalin A following autologous bone marrow transplantation (The numbers indicate the doses of bone marrow infused: $\times 10^8$ nucleated cells/kg)

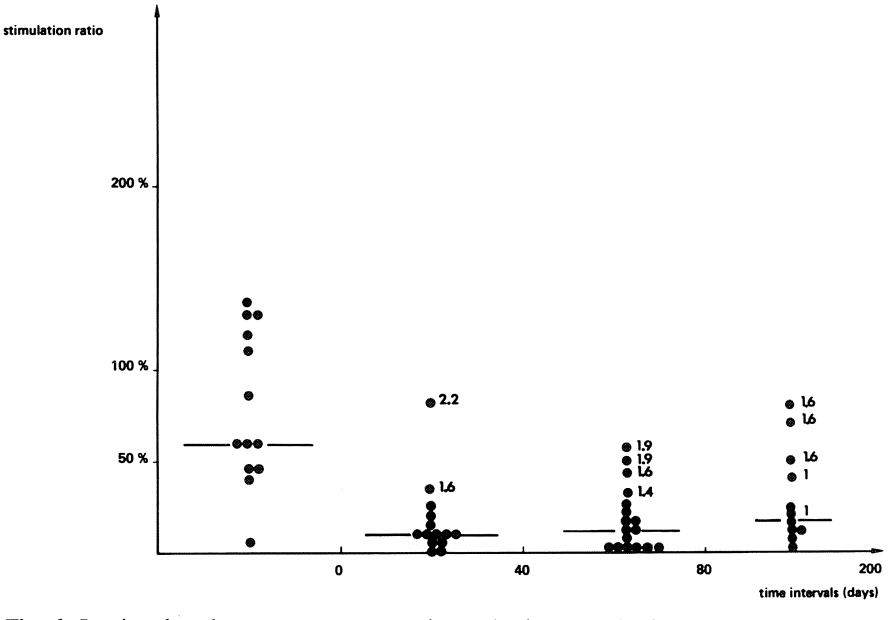


Fig. 6. In vitro lymphocyte response to pokeweed mitogen following autologous bone marrow transplantation (The numbers indicate the doses of bone marrow infused: $\times 10^8$ nucleated cells/kg)

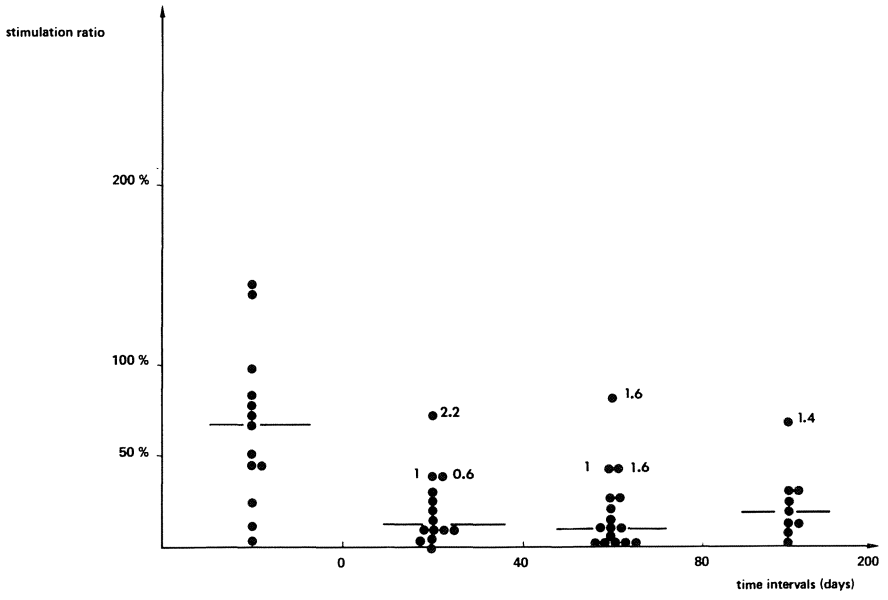


Fig. 7. MLC response following autologous bone marrow transplantation (The numbers indicate the doses of bone marrow infused: $\times 10^8$ nucleated cells/kg)

diminished, and clear stimulation in the range of pretransplant responses was obtained in many instances. Although no statistical correlation could be drawn, the degree of the responses was again related to the dose of cryopreserved marrow infused.

As expected, patients receiving TACC without cryopreserved marrow had very low levels of response.

E. Discussion

Autologous bone marrow transplantation (ABMT) is currently under investigation in numerous institutions. Both the efficacy of bone marrow cryopreservation on the haematopoietic stem cell viability and the reality of autologous grafting following the infusion of cryopreserved marrow have been demonstrated in man (Appelbaum et al., 1978b; Schaefer et al., 1977; Buckner et al., 1978; Gorin et al., 1979). Current clinical trials have suggested that ABMT may be useful in the management of patients with acute leukemia (Dicke et al., 1979; Schaefer et al., 1977; Gorin et al., 1979), chronic myelocytic leukemia in blast crisis (Buckner et al., 1978; Goldman et al., 1978), malignant lymphoma including Burkitt's lymphoma (Appelbaum et al., 1978a) and some other solid tumors (Tobias et al., 197 Gorin et al., 1978; Graze et al., 1979; Kaiser et al., 1979).

It is therefore predictable that the use of ABMT to allow higher dosage of chemotherapy and/or radiotherapy for the treatment of malignant tumors will be developed in the coming years.

However these maneuvres may induce dangerous side effects and the immune status of the patients following ABMT should be carefully studied in view of the possible risks of immunodepression which include activation of latent viruses (Rinaldo Jr. et al., 1976) and tumor growth enhancement, even though massive tumor cytoreduction has previously been obtained with high dose chemotherapy and/or TBI.

In addition, autologous bone marrow transplantation provides a unique experimental model of immune reconstitution in supposedly optimal conditions where host-donor interactions, such as graft rejection and graft versus host reaction, do not occur. We thought that studying immunocompetence in this setting might provide interesting data.

The results of our study in patients submitted to autologous bone marrow transplantation seem roughly similar to the results obtained in patients submitted to allogeneic transplantation (Halterman et al., 1972; Fass et al., 1973; Bleyer et al., 1975; Elfenbein et al., 1976; Noel et al., 1976; Gale et al., 1978; Witherspoon et al., 1978; Neely et al., 1978). Quantitative determinations of antibodies against viral and bacterial antigens showed no decrease from pretransplant levels. Some patients responded adequately to CMV and herpes virus infection and 2 cases of primary spontaneous immunization were observed. Lymphocyte counts in peripheral blood returned to pretransplant values within 6 weeks, but their reactivity to PHA and Con A remained profoundly depressed up to 200 days post-transplant. Interestingly, the reactivity to pokeweed mitogen and in mixed lymphocyte cultures was better and in some instances returned rapidly to pretransplant levels.

The similarity of the immune status following autologous and allogeneic bone marrow transplantation is however not complete: patients receiving cryopreserved autologous marrow in our series were grafted with doses of bone marrow 2 to 10 times lower than the doses of marrow usually infused for allogeneic transplantation.

Since we observed a relation of the kinetics of immune reconstitution to the dose of autologous marrow infused, it is conceivable that, for identical doses of marrow, the kinetics indeed would be much better with autologous marrow. Also, we must point out that the incidence of interstitial pneumonitis in our series was very low (1 pneumocystis carinii infection) compared with the 50% incidence following allogeneic bone marrow transplantation (Thomas et al., 1977).

Although only preliminary, we think that our data could suggest the following conclusions:

1. Although the minimum dose for autologous bone marrow transplantation in man is probably around $0.5\ 10^8$ nucleated cells/kg, much higher doses should be used to ensure faster and better restoration of immune reactivity. This might be particularly relevant to patients with solid tumors and lymphomas in contrast to patients with acute leukemia, for whom minimum doses of marrow harvested in complete remission may on the contrary hopefully provide only minimum numbers of contaminating leukemic cells.
2. The similarity of the immunological dysfunction following autologous and allogeneous bone marrow transplantation suggest that in the immediate post-transplant period, the role of the conditioning regimen is predominant,

whereas the role of GVHD in cellular immunity depression may be minimal. A longer follow-up study of patients submitted to autologous transplantation may help to define more clearly the role of GVHD in the prolonged immunocompetence following allogeneic bone marrow transplantation.

Acknowledgements

The authors are grateful to Mrs. O. Prou and Mrs. A. M. d'Ambrosio for skilful technical assistance and to Mrs. M. Veillard for excellent secretarial assistance.

References

Appelbaum, F. R., Deisseroth, A. B., Graw, R. G., Herzig, G. P., Levine, A. S., Magrath, I. T., Pizzo, P. A., Poplack, D. G., Ziegler, J. L.: Prolonged complete remission following high dose chemotherapy of Burkitt's lymphoma in relapse. Cancer *41,* 1059–1063 (1978a)

Appelbaum, F. R., Herzig, G. P., Ziegler, J. L., Graw, R. G., Levine, A. S., Deisseroth, A. B.: Successful engraftment of cryopreserved autologous bone marrow in patients with malignant lymphoma. Blood *52,* 85–95 (1978b)

Bleyer, W. A., Blaese, R. M., Bujak, J. S., Herzig, G. P., Graw, R. G.: Long term remission from acute myelogenous leukemia after bone marrow transplantation and recovery from acute graft versus host reaction and prolonged immunoincompetence. Blood *45,* 171–181 (1975)

Buckner, C. D., Stewart, R., Clift, R. A., Fefer, A., Neiman, P. E., Singer, J., Storb, R., Thomas, E. D.: Treatment of blastic transformation of chronic granulocytic leukemia by chemotherapy, total body irradiation and infusion of cryopreserved autologous marrow. Exp. Hematol. *6,* 96–109 (1978)

Dicke, K. A., McCredie, K. B., Spitzer, G., Zander, A., Peters, L., Verma, D. S., Stewart, D., Keating, M., Stevens, E. E.: Autologous bone marrow transplantation in patients with adult acute leukemia in relapse. Transplantation *26,* 169–173 (1978)

Dicke, K. A., Stevens, E. E., Spitzer, G., McCredie, K. B., Bottino, J.: Autologous bone marrow transplantation in adult acute leukemia. Exp. Hematol. (abstract) *5,* 105 (1977)

Dicke, K. A., Zander, A., Spitzer, G., Verma, D. S., Peters, L., Vellekoop, L., McCredie, K. B.: Autologous bone marrow transplantation: Adult acute leukemia in relapse. Preprint (1979)

Elfenbein, G. J., Anderson, P. N., Humphrey, R. L., Mullins, G. M., Sensenbrenner, L. L., Wands, J. R., Santos, G. W.: Immune system reconstitution following allogeneic bone marrow transplantation in man: a multiparameter analysis. Transplant. Proc. *8,* 641–646 (1976)

Fass, L., Ochs, H. D., Thomas, E. D., Mickelson, E., Storb, R., Fefer, A.: Studies of immunological reactivity following syngeneic or allogeneic marrow grafts in man. Transplantation *16,* 630–640 (1973)

Gale, R. P., Opelz, G., Mickey, M. R., Graze, P. R., Saxon, A.: Immunodeficiency following allogeneic bone marrow transplantation. Transplant. Proc. *10,* 223–227 (1978)

Goldman, J. M., Th'ng, K. H., Park, D. S., Spiers, A. S. D., Lowenthal, R. M., Ruutu, T.: Collection, cryopreservation and subsequent viability of haemopoietic stem cells intended for treatment of chronic granulocytic leukaemia in blast cell transformation. Br. J. Haematol. *40,* 185–195 (1978)

Gorin, N. C., Elgjo, R., Stout, F., Knutsen, T.: Long term preservation of canine bone marrow: in vitro studies. Blood Cells *4,* 419–429 (1978c)

Gorin, N. C., Herzig, G., Bull, M. I., Graw, R. G.: Long term preservation of bone marrow and stem cell pool in dogs. Blood *51,* 257–265 (1978a)

Gorin, N. C., Najman, A., David, R., Stachowiak, J., Hirsch-Marie, F., Muller, J. Y., Petit, J. C., Leblanc, G., Parlier, Y., Jullien, A. M., Cavalier, J., Salmon, C., Duhamel, G.: Autogreffe de moelle osseuse après chimiothérapie lourde. Nouv. Presse Med. *7,* 4105–4110 (1978b)

Gorin, N. C., Najman, A., Duhamel, G.: Autologous bone marrow transplantation in acute myelocytic leukemia. Lancet *1977 I,* 1050

Gorin, N. C., Najman, A., Salmon, Ch., Muller, J. Y., Petit, J. C., David, R., Stachowiak, J., Hirsch-Marie, F., Parlier, Y., Duhamel, G.: High dose combination chemotherapy (TACC) with and without autologous bone marrow transplantation for the treatment of acute leukemia and other malignant diseases. Kinetics of recovery of haemopoiesis. A preliminary study of 12 cases. Eur. J. Cancer, in press (1979)

Graze, P. R., Wells, J. R., Ho, W., Gale, R. P., Cline, M. J.: Successful engraftment of cryopreserved autologous bone marrow stem cells in man. Preprint (1979)

Halterman, R. H., Graw, R. G., Fucillo, D. A., Leventhal, B. G.: Immunocompetence following allogeneic bone marrow transplantation in man. Transplantation *14*, 689–697 (1972)

Kaiser, H., Leventhal, B. G., Wharam, M. D., Munoz, L. L., Elfenbein, G. J., Tutschka, P. J., Santos, G. W.: Cryopreserved autologous bone marrow transplantation in the treatment of selected pediatric malignancies: a preliminary report. Preprint (1979)

Neely, J. E., Neely, A. N., Kersey, J. H.: Immunodeficiency following human marrow transplantation: in vitro studies. Transplant. Proc. *10*, 229 (1978)

Noel, D., Storb, R., Atkinson, K., Ochs, H., Weiden, P., Fefer, A., Thomas, E. D.: Studies of immunologic reactivity in 62 human long term survivors after marrow transplantation. Blood (Abstract) *48*, 989 (1976)

Rinaldo Jr. C. R., Hirsch, M. S., Black, P. H.: Activation of latent viruses following bone marrow transplantation. Transplant. Proc. *8*, 669–672 (1976)

Schaefer, U. W.: Transplantation of fresh allogeneic and cryopreserved autologous marrow in acute leukemia. 6th annual conference of ISEH (Abstract). Exp. Hematol. *5*, 101 (1977)

Thomas, E. D., Buckner, C. D., Banaji, M., Clift, R. A., Fefer, A., Flournoy, N., Goodell, B. W., Hickman, R. O., Lerner, K. G., Neiman, P. E., Sale, G. E., Sanders, J. E., Singer, J., Stevens, M., Storb, R., Weiden, P. L.: One hundred patients with acute leukemia treated by chemotherapy, total body irradiation and allogeneic marrow transplantation. Blood *49*, 511–533 (1977)

Tobias, J. S., Weiner, R. S., Griffiths, C. T., Richman, C. M., Parker, L. M., Yankee, R. A.: Cryopreserved autologous marrow infusion following high dose cancer chemotherapy. Eur. J. Cancer *13*, 269–277 (1977)

Witherspoon, R., Noel, D., Storb, R., Ochs, H. D., Thomas, E. D.: The effect of graft versus host disease on reconstitution of the immune system following marrow transplantation for aplastic anemia or leukemia. Transplant. Proc. *10*, 233–235 (1978)

Discussion

Haas: The critical point is that you did not use any irradiation of the leukemic patient, so you cannot rule out spontaneous recovery of the patient's lympho-hemopoietic system. 3 patients, as I understood, were not transplanted and survived.

Gorin: We had 4 control patients who received the same high dose combination chemotherapy and no cryopreserved marrow. One died on day 15, 3 remained alive and slowly recovered their peripheral blood counts within 32 days in comparison to 15 days in the group of patients receiving marrow.

Netzel: In your canine and human studies, have you made any estimate on the minimum number of CFU-C necessary for engraftment?

Gorin: No, we have not.

Dicke: A kind of safe number of CFU-C to get a take in our hands is about 5000/kg. An important point is that our plating efficiency of normal bone marrow is not very high, about $15–20 \times 10^5$ cells. We use placenta conditioned medium as a stimulus which gives very reproducible results. The numbers are in confirmation with our monkey data where the minimum number of CFU-C was about 20,000, with a 5 times higher plating efficiency.

Autologous Bone Marrow Transplantation. The Influence of Prolonged Cytotoxic Chemotherapy

U. W. Schaefer, M. R. Nowrousian, S. Öhl, W. R. Boecker, M. E. Scheulen, B. Schilcher, and C. G. Schmidt

A. Introduction

In 1977 our group performed an autologous bone marrow transplantation in a patient suffering from acute myeloid leukemia [12, 13]. The bone marrow to be grafted had been harvested and cryopreserved during remission after one year of maintenance therapy. Although an adequate number of vital in vitro colony forming cells (CFU-C) was injected, the hemopoietic capacity of the transplant appeared to be deficient. In spite of an early take of the graft and a rapid repopulation of the marrow space the hemopoiesis was not able to counteract efficiently recurrent infections.

From this clinical observation we concluded that the transplantation potential of cryopreserved remission marrow frozen after long term chemotherapy might be impaired. To elucidate the problem if repeated cytotoxic therapy causes permanent defects of the proliferative capacity of bone marrow, we investigated the fluctuations of colony forming units in vitro (CFU-C) and in the mouse spleen (CFU-S) as well as the proliferative activity in diffusion chamber (DC) in mice treated with weekly injections of cyclophosphamide. In addition CFU-C and proliferation in DC were measured in bone marrow of patients receiving prolonged cytotoxic chemotherapy.

B. Materials and Methods

I. Autologous Bone Marrow Transplantation

A 38 years old man suffering from acute myeloid leukemia was grafted with cryopreserved autologous bone marrow. With daunomycin and cytosine arabinoside a complete remission could be induced. The remission was maintained by ARA-C (200 mg/m^2/d$\times$5d i.v., every 4 weeks), in addition a second drug was given which alternated every month (thioguanine 200 mg/m^2/d$\times$5d per os, cyclophosphamide 800 mg/m^2/d$\times$1d i.v., CCNU 100 mg/d$\times$1d per os, daunomycin 45 mg/m^2/d$\times$2d i.v.). The marrow was harvested during the first remission after one year of maintenance chemotherapy, 6 weeks after the last therapy course. The interval from cryopreservation to the first relapse was 6 months. The marrow was frozen in 10% DMSO and kept in liquid nitrogen for 15 months. The retransfusion was performed during the second relapse when the disease appeared to be resistant to reinduction therapy. The conditioning regimen before transplantation consisted of cyclophosphamide (50 mg/kg/d$\times$2d i.v.) and total body irradiation (860 rad at 15 rad/min, linear accelerator). Twenty-four hours after irradiation the marrow was thawed, diluted, centrifuged, filtrated and transfused i.v. 2.0×10^8 nucleated cells per kg body weight containing approximately 30,000 CFU-C per kg b.w. were administered.

II. Cryopreservation Technique

Our cryopreservation studies have been published in detail elsewhere [9–13]. As protective medium 10% DMSO was used supplemented by 20% calf serum or autologous serum. Cell concentration during freezing was 20×10^6 to 100×10^6 per ml. The cells were frozen in plastic bags or ampoules at a low freezing rate of 1–2° C/min and then kept in liquid nitrogen. After thawing in a water bath of 40–50° C the cell suspensions were slowly diluted in Hanks Balanced Salt Solution (HBSS) in order to avoid an osmotic cell death. Increasing volumes of HBSS were added stepwise until a 10-fold dilution was reached by 45 min.

III. Assays

For the in vitro culture of human hemopoietic stem cells committed to granulopoiesis (CFU-C) a double layer agar system was utilized which is a modification of the technique described by Pike and Robinson [8]. The culture medium consisted of agar, Dulbeccos medium and horse serum. Usually $0{,}2 \times 10^6$ bone marrow buffy coat cells were cultured per plate. The cultures were set up in duplicates and incubated for 14 days at 37° C, 100% humidity and 10% CO_2. Colonies were defined as cell aggregates of more than 50 cells.

In mice the agar culture technique described by Dicke was used [3]. As source of colony stimulating activity embryonic fibroblasts were added to the underlayer. Duplicate cultures were evaluated after 7 days of incubation.

The diffusion chamber technique for growth of murine or human bone marrow was utilized as described by Cronkite et al. [2].

For the in vivo determination of hemopoietic stem cells in mice the spleen colony technique of Till and McCulloch was used [14]. Donors and recipients were female F_1 (CBA × C57 BL) hybrid mice.

IV. Chemotherapy Regimens

Induction and maintenance therapy in acute leukemia have been described above. In testicular terato carcinoma the chemotherapy consisted of courses of vinblastine (0,2 mg/kg/d × 2d i.v.) plus bleomycin (0,4 mg/kg/d × 5d i.v. continuous infusion) and adriamycin (60 mg/m²/d × 1d i.v.) plus cis-platinum (20 mg/m²/d × 5d i.v.) The therapy courses were administered at monthly intervals for one year. Two courses of vinblastine/bleomycin alternated with two courses of adriamycin/cis-platinum.

In mice bone marrow donors were injected i.p. at weekly intervals with cyclophosphamide (80 mg/kg b.w.) for 41 weeks. Again F_1(CBA × C57 BL) female hybrids were used.

V. Patients

In leukemia patients (acute non-lymphocytic leukemia) every month marrow aspirations were performed just before the regular maintenance chemotherapy course. Patients who went into leukemic relapse within 8 weeks after the examination were excluded from the evaluation.

The patients with testicular terato carcinoma were investigated in the same way. In this group there was no patient with bone metastasis or bone marrow involvement.

In both groups of patients the CFU-C studies were performed with fresh bone marrow cells. In diffusion chambers cryopreserved marrow samples were examined.

C. Results

I. Autologous Bone Marrow Transplantation

The clinical course of the patient who received frozen autologous bone marrow is demonstrated in Figure 1. A rapid take was proved by increase of leukocytes, reticulocytes and platelets soon after the supralethal conditioning. Already 60

days after transplantation bone marrow biopsies revealed an almost normal cellularity. Two months after grafting infections occured and in spite of the good repopulation of the marrow space the peripheral blood cells decreased again. Finally the patient died with peripheral cytopenia from cerebral hemorrhage.

II. Acute Leukemia

In adult patients with acute leukemia in remission maintained by monthly chemotherapy courses the mean CFU-C content showed a steady decline during one year of therapy (Table 1). The difference found by comparison of the mean values measured in the first quarter and the last quarter of the 12 months phase is significant.

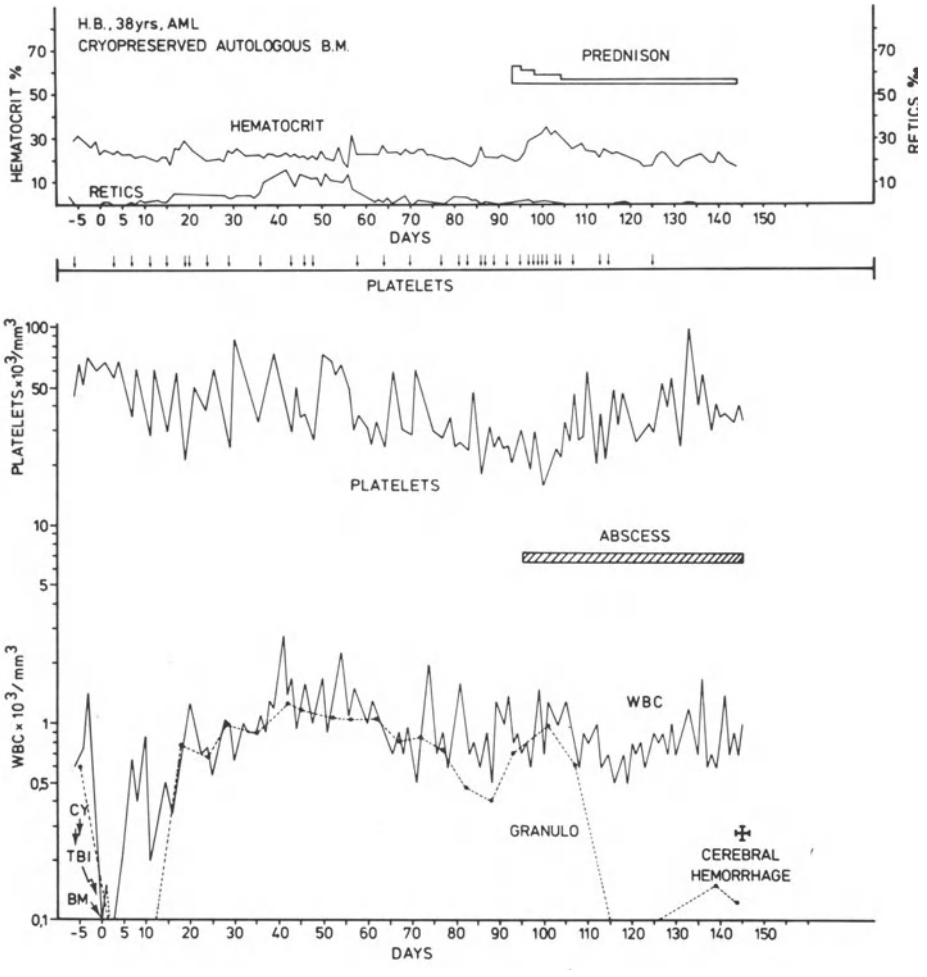


Fig. 1

Remission duration (weeks)	Bone marrow CFU-C (means ± SE) per 10^5 nucleated cells
Up to 12 weeks	38 ± 4,7[a]
12 to 24 weeks	29 ± 4,8
24 to 36 weeks	24 ± 7,7
36 to 48 weeks	21 ± 3,5[a]

[a] The difference between the first and the last group is significant ($p<0,05$)

Table 1. CFU-C numbers in remission of acute non-lymphocytic leukemia in relation to the duration of complete remission

III. Testicular Carcinoma

In testicular terato carcinoma a different pattern was found. The mean CFU-C content did not decline significantly during one year of cyclic chemotherapy (Fig. 2). On the other hand the diffusion chamber results revealed a tendency that repeated courses of chemotherapy decrease the cell number produced during DC culture. The more therapy courses were administered the lower the values for total cell numbers per chamber were found (Fig. 3).

IV. Studies in Mice

The results of the studies in mice are shown in Fig. 4 and Table 2. There was no significant decrease of CFU-S numbers per femur during 41 weeks of therapy. From week 7 to week 13 and again in week 33, slightly more CFU-S were found in

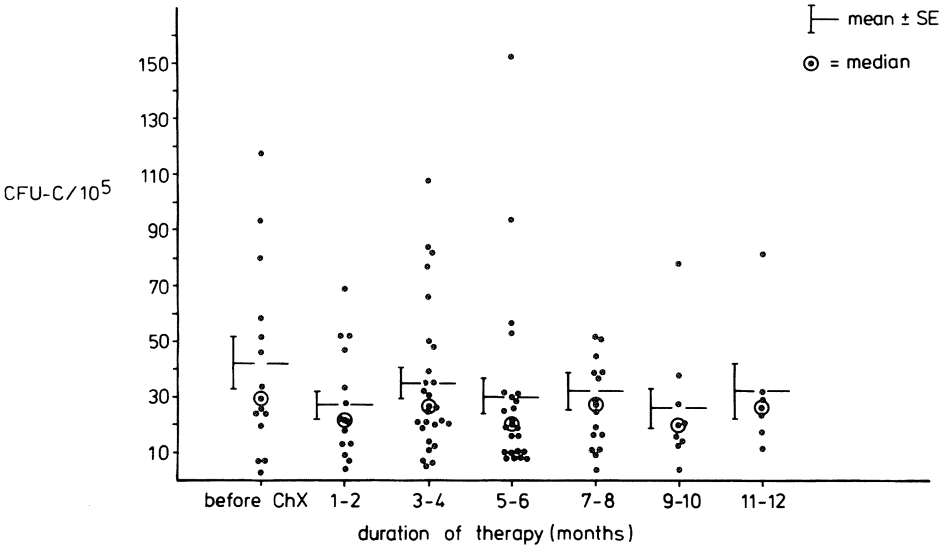


Fig. 2. CFU-C in bone marrow of terato carcinoma patients during chemotherapy

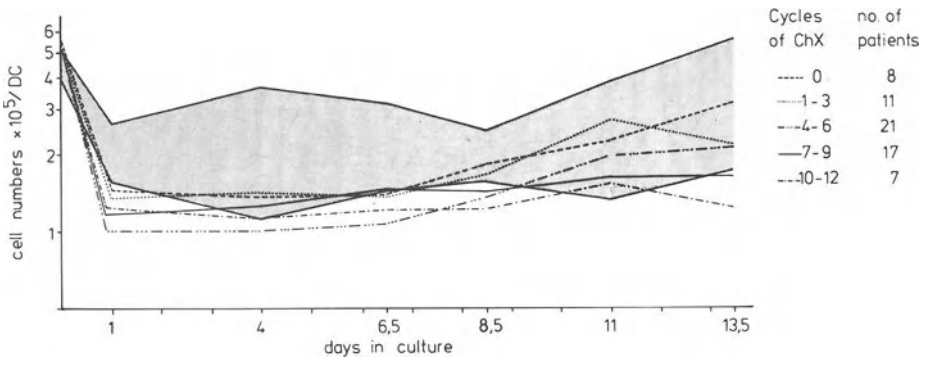


Fig. 3. Bone marrow DC cultures in patients with terato carcinoma

	CTX treatment[b]	Control
Total cells/DC	1,077,000	550,000
CFU-S/DC	194	68
CFU-C/DC	528	48

[a] For each parameter the content of 4 chambers was evaluated. The listed numbers are mean values of a single experiment

[b] The assays were performed 10 weeks after discontinuation of the longterm cyclophosphamide treatment (80 mg/kg, at weekly intervals i.p. for 41 weeks)

Table 2. Total cell recovery and content of CFU-S and CFU-C per chamber after 9 days of diffusion chamber culture[a]

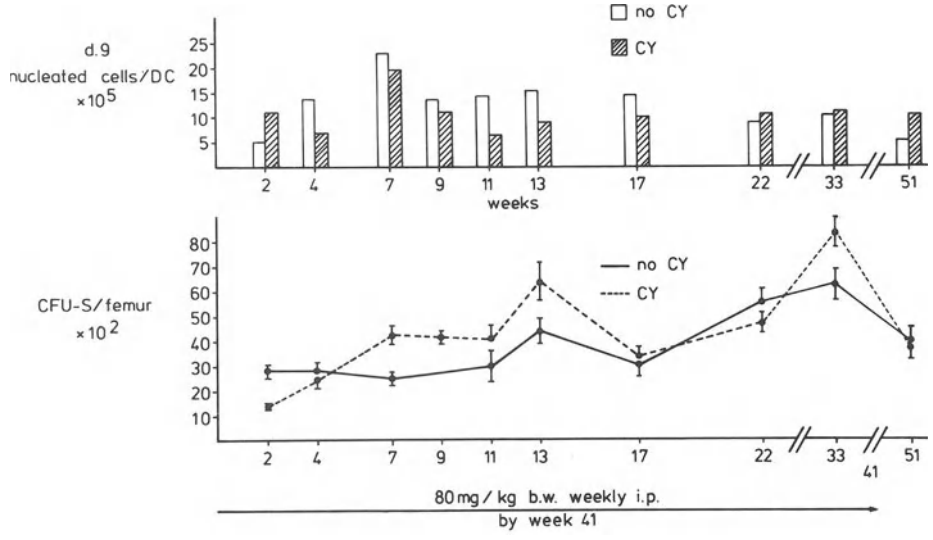


Fig. 4. CFU-S per femur and nucleated cells per diffusion chamber during cyclophosphamide treatment

the cyclophosphamide group than in the untreated controls. Cell recovery in DC did not differ significantly in the two groups during observation time. Cryopreserved marrow samples tested incidentally gave similar results. Ten weeks after discontinuation of the cyclophosphamide treatment CFU-S, CFU-C and total cell content in diffusion chambers after 9 days of culture were measured. In the cyclophosphamide group 2,8 times more CFU-S, 11 times more CFU-C and 1,7 times more total cells were found at day 9. Without passage trough the diffusion chamber culture the CFU-S values in treated and untreated mice were identical (Fig. 4). The results of the passage experiment are listed in Table 2. These are preliminary data of a single experiment.

D. Discussion

In previous reports we have demonstrated that human bone marrow of normal individuals and leukemia patients in remission can be stored at low temperature without significant loss of proliferative activity [9, 10, 11, 13]. Our first attempt to apply cryopreserved cells in clinical bone marrow transplantation demonstrated that high proliferative activity in agar and in diffusion chamber culture as well as an early take of the frozen-thawed transplant do not necessarily predict a prompt hemopoietic restoration. Under normal conditions the number of CFU-C correlates well to the transplantation potential of mouse and primate bone marrow [4]. This might be different however when the stem cell population is perturbated by repeated courses of myelosuppressive cytotoxic therapy.

In a number of studies hemopoietic progenitor cells (CFU-S, CFU-C, CFU-E) have been measured under the effect of different cytotoxic agents given as single massive dose. Reports which deal with the effect of long term chemotherapy on colony forming cells are rare [1,5–7]. Pannacciulli et al [7] studied CFU-S, CFU-C and CFU-E in mice given cyclophosphamide for 18 weeks in small daily doses. After 18 weeks of treatment no reduction of CFU-S, CFU-C and CFU-E per femur was found. CFU-S and CFU-E were at the end point near to normal, CFU-C was increased to 2–3 times the control values.

Hellman and coworkers performed serial transplantations in mice pretreated with cytotoxic agents for 5–6 weeks [1, 5]. Their results support the theory that the hemopoietic renewal system has limited capacity. After busulfan a marked, after L-phenylalanine mustard a moderate, after cyclophosphamide no significant damage of bone marrow stem cells were observed.

Lohrmann et al. reported serial studies of granulopoiesis during and after intermittent adjuvant chemotherapy for breast cancer (adriamycin plus cyclophosphamide, given for six courses at monthly intervals) [6]. Following discontinuation of chemotherapy a long lasting reduction of peripheral neutrophiles and CFU-C in bone marrow and peripheral blood were found.

We studied in two different groups of patients the effect of prolonged intermittent polychemotherapy on bone marrow CFU-C. In the first group the hemopoiesis had a malignant defect, in the second group the hemopoietic system was not involved by the tumor. In leukemia, a steady decline of CFU-C was measured in relation to the duration of remission and the number of therapy courses. Patients with terato carcinoma did not exhibit a significant reduction of

bone marrow CFU-C during 12 cycles of chemotherapy. In DC cultures however a reverse correlation between proliferation activity and numbers of therapy courses was found for the latter group of patients, but the differences were not statistically significant.

In mice treated for prolonged periods of time with moderate doses of cyclophosphamide neither the CFU-S content nor the total cell recovery in DC decreased significantly. Although 10 weeks after discontinuation of cyclophosphamide bone marrow CFU-S of treated and control animals did not differ, a significant perturbation of hemopoiesis became apparent when CFU-S and CFU-C were tested after marrow passage through the diffusion chamber culture. After 9 days in DC culture the marrow of cyclophosphamide treated mice produced 1,7 times higher cell count, 2,8 times more CFU-S and 11 times more CFU-C per DC than the controls. Apparently the marrow of the treated mice was primed to go rapidly into active proliferation under the conditions of the diffusion chamber culture. These preliminary results indicate that the DC method can be used as amplification system to make perturbation of the hemopoiesis detectable which the conventional CFU assays fail to demonstrate.

Our ongoing experiments and the results of other investigators mentioned above, indicate that the pattern of perturbation after chemotherapy is very much dependent on the agent, the dose and the application schedule. Therefore one cannot conclude that the transplantation potential of bone marrow is always reduced when chemotherapy has been administered for prolonged periods of time. One has to keep in mind however, that certain drugs can cause long lasting perturbations of hemopoiesis which sometimes cannot be detected by the conventional CFU assays.

In leukemia the hemopoietic disorder itself, the limited self renewel capacity of the stem cell population stressed in addition by repeated courses of chemotherapy and possible long lasting defects of hemopoiesis due to certain cytotoxic agents might hamper autologous bone marrow transplantation.

Because of these clinical and experimental data we decided to harvest bone marrow for transplantation in future during early remission when only a few therapy courses have been administered.

E. Summary

A patient suffering from relapse of AML was grafted with cryopreserved autologous bone marrow harvested during remission. Before transplantation the patient was treated with a supralethal cytoreductive chemo-radiotherapy. There was a rapid take and a repopulation of the marrow space but the hemopoiesis appeared to be deficient to counteract infections.

Bone marrow aspirates of patients with acute non-lymphocytic leukemia and of patients with testicular carcinoma were tested for CFU-C. In leukemia a steady decline of CFU-C was measured in relation to the duration of remission and the numbers of chemotherapy courses. In terato carcinoma no significant decrease of CFU-C could be observed. A reverse correlation between numbers of therapy courses and proliferation activity in diffusion chambers was found in this group of patients, but the differences were not significant.

In mice, prolonged cyclophosphamide treatment (80 mg/kg i.p., given at weekly intervals for 41 weeks) did not decrease the CFU-S content per femur nor the cell recovery in the diffusion chamber culture, but 10 weeks after cessation of therapy a significant perturbation of the hemopoiesis in the treated mice could still be detected when CFU-S and CFU-C were measured in cell suspensions which had been cultured 9 days in diffusion chamber cultures.

Acknowledgements

The skilful help of Mrs. Liane Baatsch and Mr. Rolf Mathes and of many members of the bone marrow transplantation team is gratefully appreciated. The work was supported by Landesamt für Forschung, Düsseldorf, Deutsche Forschungsgemeinschaft (SFB Essen), and Bundesministerium für Jugend, Familie und Gesundheit.

References

1. Botnick, L. E., Hannon, E. C., Hellman, S.: Multisystem stem cell failure after apparent recovery from alkylating agents. Cancer Res. *38,* 1942–1947 (1978)
2. Cronkite, E. P., Carsten, A. L., Chikkappa, G., Laissue, J. A., Öhl, S.: Culture of normal and leukemic cells in diffusion chambers. In: Advances in the Biosciences 14. Fliedner, T. M., Perry, S. (eds.), pp. 273–286. Oxford, New York, Toronto, Sydney: Pergamon Press 1973
3. Dicke, K. A., Platenburg, M. G. C., Bekkum, D. W. van: Colony formation in agar: in vitro assay for haemopoietic stem cells. Cell Tissue. Kinet. *4,* 463–477 (1971)
4. Dicke, K. A., Noord, M. J. van, Maat, B., Schaefer, U. W., Bekkum, D. W. van: Identification of cells in primate bone marrow resembling the hemopoietic stem cell in the mouse. Blood *42,* 195–208 (1973)
5. Hellman, S., Botnick, L. E., Hannon, E. C., Vigneulle, R. M.: Proliferative capacity of murine hematopoietic stem cells. Proc. Natl. Acad. Sci. USA *75,* 490–494 (1978)
6. Lohrmann, H.-P., Schreml, W., Lang, M., Betzler, M., Fliedner, T. M., Heimpel, H.: Changes of granulopoiesis during and after chemotherapy of breast cancer. Br. J. Haematol. *40,* 369–381 (1978)
7. Pannaccciulli, I. M., Massa, G., Saviane, A. G., Biachi, G., Bogliolo, G. V., Ghio, R.: The effects of chronic administration of cyclophosphamide on haemopoietic stem cells. Scand. J. Haematol. *19,* 217–223 (1977)
8. Pike, B. L., Robinson, W. A.: Human bone marrow colony growth in agar-gel. J. Cell. Physiol. *76,* 77–84 (1970)
9. Schaefer, U. W., Dicke, K. A., Bekkum, D. W. van: Recovery of haemopoiesis in lethally irradiated monkeys by frozen allogeneic bone marrow grafts. Rev. Europ. Etud. Clin. Biol. *17,* 483–488 (1972)
10. Schaefer, U. W., Dicke, K. A.: Preservation of haemopoietic stem cells. Transplantation potential and CFU-C activity of frozen marrow tested in mice, monkeys and man. In: La cryoconservation des cellules normales et neoplasiques; Weiner, R. S., Oldham, R. K., Schwarzenberg, L. (eds.), pp. 63–69. Paris: Éditions INSERM 1973
11. Schaefer, U. W., Schmidt, C. G., Dicke, K. A., Bekkum, D. W. van, Schmitt, G.: Konservierung von hämopoetischen Stammzellen. Z. Krebsforsch. *83,* 285–291 (1975)
12. Schaefer, U. W. and coworkers: Transplantation of fresh allogeneic and cryopreserved autologous bone marrow in acute leukemia. Exp. Hematol. *5,* Suppl. 2, 101 (1977)
13. Schaefer, U. W., Nowrousian, M. R., Öhl, S., Schmidt, C. G.: Cryopreservation of bone marrow. In: Cell-separation and cryobiology, Rainer, H., Borberg, H., Mishler, J. M., Schaefer, U. W. (eds.), pp. 243–254. Stuttgart, New York: Schattauer 1978
14. Till, J. E., McCulloch, E. A.: A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. *14,* 213–222 (1961)

Discussion

Haas: When would you take bone marrow in your leukemic patient for storage?
Schaefer: I would recommend to take the marrow in the first three or four months after induction therapy.
Prindull: We are measuring CFU-C in the bone marrow of children with ALL in remission treated by either 6-mercaptopurine and methotrexate or cytoxan in addition once per week. The preliminary impression is that CFU-C do not decline over a long period of time.
Schaefer: Our treatment regimen is far more aggressive which may explain the difference.
Dicke: We have observed low CFU-C levels in the marrow of patients being off therapy after 3–4 years. The reason may be a selective group of patients or a late effect of chemotherapy on the stem cell pool.
Lohrmann: We have observed a defect of CFU-C in breast cancer patients treated with chemotherapy shortly after the start of therapy and it persists.
Gerhards: Did you study CFU-C in the blood?
Schaefer: No. I don't think that this is useful. There are few CFU-c per 10^5 blood cells and what means a difference between 1 and 10 CFU?
Kersey: You and others have seen a primiry effect of cyclophosphamide, is this effect unique to cyclophosphamide or also seen with other cycle dependent agents?
Schaefer: We only tested this agent so far, but similar effects have been shown with other agents by the Hellman group.
Dicke: One has to be very sceptical of CFU-S assays, since it depends on the seeding efficiency in the spleen. More resistant subpopulations surviving chronic treatment with cyclophosphamide may be selected. This may explain the discrepancy between CFU-S and diffusion chamber assays, therefore survival data are important to be added.
Lohrmann: In a similar study of Hellman the CFU-S pool per femur decreases after treatment with cyclophosphamide, whereas in your study the proliferative capacity of CFU-S in the diffusion chamber increases. Can you explain the discrepancy?
Schaefer: The duration of therapy is different, Hellman gave only five or six courses, we treated for a longer time.

Requirements for the Sucessful Application of Autologous Bone Marrow Transplantation in the Treatment of Selected Malignancies*

H. Kaizer, M. D. Wharam, R. J. Johnson, J. G. Economou, H. S. Shin, G. W. Santos, G. J. Elfenbein,** P. J. Tutschka, H. G. Braine, L. L. Munoz, and Brigid G. Leventhal

A. Introduction

Bone marrow suppression is often the critical factor limiting the intensity of cytotoxic therapy which can be administered to patients with cancer [7]. Autologous bone marrow transplantation can be used to circumvent this dose limiting toxicity, while avoiding some of the problems encountered in allogeneic bone marrow transplantation [16]. The successful application of autologous bone marrow transplantation to the treatment of cancer depends on three requirements. First, it must be possible to obtain and viably store sufficient numbers of hematopoietic stem cells to ensure recovery of hematologic and immunologic function. Second, clonogenic tumor cells must be absent from the marrow cell inoculum. Third, the tumor remaining in the patient must be sensitive to an intensive pulse of pretransplant therapy at doses which have acceptable extramedullary toxicity. Detailed accounts of prior studies of autologous marrow transplantation and the problems and possible benefits of the procedure can be found in three recent reviews [5, 8, 23].

We have begun clinical trials at The Johns Hopkins Oncology Center on the use of cryopreserved autologous bone marrow reinfusions for the treatment of selected malignancies. Since studies involving autologous marrow grafts provide no markers to conclusively document engraftment, our preliminary treatment protocols have been designed to deliver known marrow-lethal doses of cytotoxic therapy so that the strongest possible evidence could be obtained that the patient's hematologic and immunologic recovery was due to reinfused stem cells. This paper will present data on the first seven patients to have received autologous marrow transplants in our studies. In addition, we will present some clinical observations and animal studies relating to the question of tumor cells contaminating reinfused marrow. Portions of this work have been previously reported [6, 9].

B. Materials and Methods

I. Clinical studies

Patients eligible for study include all patients over the age of 2 years with poor-risk acute leukemia, stage III or IV neuroblastoma, stage III or IV non-Hodgkin's lymphoma, extensive small cell carcinoma of the lung or Stage IV rhabdomyosarcoma. All patients initially receive conventional

* Supported by grants (CA-06973 and CA-15396) of the National Cancer Institute, DHEW.

** Dr. Elfenbein is an investigator of the Howard Hughes Medical Institute.

chemotherapy for their disease except for patients with small cell carcinoma of the lung who are transplanted per-primum. Bone marrow is harvested for storage after remission induction therapy if multiple bone marrow aspirates and biopsies immediately prior to harvesting show no microscopically detectable foci of tumor. Collection of bone marrow cells by multiple bone marrow aspirations under general anesthesia has been previously described [20]. Further processing of the marrow cells and the method for cryopreservation have been described by Appelbaum et al. [2]. Viability of cryopreserved marrow is determined by an assay for granulocyte and monocyte colony forming cells in tissue culture (CFU-C) as previously described [13]. Following storage of marrow, patients are continued on conventional therapy unless they fail to achieve a complete remission or unless they relapse.

Pretransplant therapy for most patients receiving autologous marrow reinfusions consists of adriamycin (ADR), 30 mg/M^2 IV daily $\times 3$, beginning 6 days prior to transplantation. Cyclophosphamide (CTX), 1800 mg/M^2 IV daily $\times 2$, is begun 5 days pretransplant. One day prior to transplantation the patients receive total body irradiation (TBI) delivered at a rate of 8.5 rads/min to a total dose of 800 rads. Patients with residual bulky disease receive fractionated regional radiotherapy immediately prior to the start of intensive pretransplant therapy. One patient with diffuse histiocytic lymphoma, whose prior therapy had included 4400 rads of mediastinal irradiation and a total cumulative dose of adriamycin in excess of 350 mg/M^2, received treatment without TBI which included busulfan at 5 mg/kg $\times 4$ and cyclophosphamide at 50 mg/kg $\times 4$ immediately followed by marrow reinfusion. The rationale for this protocol has been described elsewhere [18]. One patient with an extensive small cell carcinoma of the lung had marrow stored prior to any therapy. He then received two 5 day cycles of combination chemotherapy consisting of cyclophosphamide at 2400 mg/M^2 on day 1, adriamycin at 60 mg/M^2 on day 1, and VP-16 at 125 mg/M^2 on days 1, 3 and 5. The use of this combination chemotherapy in small cell carcinoma of the lung has been previously described [1]. Immediately following the second cycle of chemotherapy, the patient received two doses of TBI at 400 rads given every other day and followed by reinfusion of his cryopreserved marrow. We have previously reported on the rationale for the use of fractionated TBI with bone marrow transplantation [11].

During the period of aplasia following TBI, patients are kept in reverse isolation until the granulocyte count exceeds 500 cells/mm^3. They are supported with intravenous alimentation, red blood cell, platelet and leukocyte transfusions and they receive broad spectrum antibiotics if they exhibit significant fever. Daily hemograms and weekly bone marrow aspirations are done to monitor hematologic recovery. The antitumor response is assessed by standard clinical methods.

II. In Vitro Antitumor Treatment of Marrow

The 6C3HED mouse lymphoma model and the procedures used for developing antiserum and its use in treating tumor cell-marrow cell mixtures have been previously described [6].

The rat acute myelogenous leukemia (AML) model was first described by van Bekkum and his colleagues in the Brown Norway rat [24] and adapted for passage in the Lewis-Brown Norway (LBN)F_1 hybrid [25]. Tumor (LBN-AML) cells were obtained from tumor-bearing LBN rat spleens and femoral marrow as described [17, 25]. Suspensions or normal spleen and bone marrow cells were obtained by similar techniques from tumor-free LBN rats. Antiserum to LBN-AML cells was raised in C3H mice by a procedure similar to that used by Baker and Taub to raise antisera to human acute leukemia [3]. Briefly, mice were tolerized by a single intraperitoneal (IP) injection of 200 mg/kg cyclophosphamide followed in four hours by an IV inoculation of 10^8 normal LBN spleen cells. One week later they were started on an immunization schedule with IP inoculations of 10^8 LBN-AML cells in complete Freund's adjuvant given every week for four weeks. Antiserum was obtained one week after the last inoculation and was tested for reactivity against LBN-AML and normal LBN spleen and marrow cells by a microcytotoxicity assay [15]. The source of complement was identical to that used in the 6C3HED mouse lymphoma studies. The inhibitory effect of the antiserum against rat CFU-C was tested by incubating normal LBN marrow cells at 10^6 cells/ml in McCoy's 5A tissue culture medium, with varying dilutions of antiserum, at 22° C for 30 min. Complement was added for an additional 60 min. The cells were washed with tissue culture medium and readjusted to 10^6 viable cells/ml and tested for CFU-C by the same assay we use for human marrow CFU-C. Antiserum absorbtion was carried out by incubating the antiserum with 10^8 normal LBN spleen of marrow cells per ml for 60 min at 22° C. Indirect immunofluorescence (IFA) studies of reactivity of unabsorbed and absorbed anti-LBN-AML antiserum were carried out with LBN-AML and normal LBN marrow cells using a fluoroscein labelled F(ab)$_2$ anti-mouse immunoglobulin supplied by Cappel Laboratories, Inc., Cochranville, Pa.

The studies of human T-cell lymphoid malignancies employed the following reagents. The rabbit antihuman antithymocyte globulin (Ra-ATG) was kindly supplied by Dr. S. Thierfelder of the GSF Institut für Hämatologie, Munich, W. Germany and its properties have been previously described [14]. Several lots of a horse anti-human ATG (Ho-ATG) were kindly supplied by Dr. B. Loughman of The Upjohn Co., Kalamazoo, Mich. A T-cell lymphoid line that was originally derived from a patient with a T-cell lymphoid malignancy was kindly supplied by Dr. D. Mann of the National Cancer Institute, Bethesda, Md. This T-cell line is passaged in suspension culture with RPMI 1640 plus 10% fetal calf serum as the tissue culture medium. Additional T-cell lymphoblastic target cells were obtained by freezing fresh T-cell tumor cells obtained from the marrow or malignant effusions of patients with T-cell lymphoma or leukemia. The cells were frozen in autologous plasma, TC-199 and 10% DMSO. For use they were quickly thawed in a 37° C water bath and viable cells obtained from the mononuclear cell layer at the interface of a Ficoll-Hypaque gradient. Raji cells served as prototypes for B-cell lymphoblasts and were kindly supplied Dr. S. Mayasi of The Pfizer Co., Maywood, New Jersey. Absorbtion of Ho-ATG was carried out with 10^8 Raji cell/ml of a 1:25 dilution of the antiserum and incubation was as described for other antiserum absorbtion described above. Complement for all cytotoxicity tests involving human material consisted of freshly frozen rabbit serum. Cytotoxicity tests were carried out by the same microcytotoxicity assay described above. Additional cytotoxicity tests were carried out by a ^{51}Cr release assay [12]. The CFU-C assay was identical to that described in our clinical studies.

C. Results and Discussion

I. Clinical studies

Table 1 summarizes the data on cyropreserved bone marrow obtained from 18 patients. The recovery of viable hematologic progenitor cells, as measured by CFU-C on thawed cryopreserved material, ranges from 32 to 85%, averaging 54%.

While no available method can guarantee the absence of clonogenic tumor cells in the bone marrow of patients with disseminated malignancies, all our patients except for those with extensive small cell carcinoma of the lung receive several courses of chemotherapy prior to marrow storage to reduce the number of tumor cells that might have been present in the marrow. We have previously shown that such prior chemotherapy does not necessarily reduce the numbers of marrow CFU-C's obtainable [9].

The tumors that have been selected for this study all show significant sensitivity to cytotoxic drugs and radiotherapy although they are infrequently cured. The pretransplant therapeutic regimen was selected in an attempt to maximize antitumor therapy and to deliver known marrow-lethal doses of cytotoxic therapy. CTX and ADR were selected because of their broad spectrum of antitumor activity and because their combined use in L1210 leukemia has been shown to be synergistic [22]. The dose and rate of TBI was selected because of its

Table 1. Summary of Cryopreserved Marrow (18 Patients)

	Mean	Range
Nucleated marrow cells/KG	3.5×10^8	$0.5–10.0 \times 10^8$
Total CFU-C Prefreezing	8.4×10^5	$1.8–25.0 \times 10^5$
Total CFU-C	4.9×10^5	$0.9–9.2 \times 10^5$
% recovery	54%	32–84%

tolerable extramedullary toxicity in allogeneic bone marrow transplantation [21] and because its marrow-lethal effects have been documented [4].

Thus far, seven patients have completed therapy with autologous marrow reinfusion and are evaluable. Table 2 summarizes their pretransplant characteristics, quantities of marrow infused, hematologic recovery and antitumor effects. Although each of the patients had advanced and disseminated malignancies, only patient No. 5 had microscopic evidence of marrow involvement at any time. Two patients had achieved only a partial response and four patients showed progression on relatively intensive but conventional combination chemotherapy. One patient with extensive small cell carcinoma of the lung was transplanted as part of his primary therapy. All patients started pretransplant therapy with significant residual tumor mass present. Patients Nos. 1, 2, 4 and 5 had additional regional radiotherapy to areas of bulky disease immediately prior to the start of systemic cytotoxic therapy. In patient No. 2, the entire thorax was shielded during TBI because the regional radiotherapy pretreatment to the entire left hemithorax posed a significant risk of radiation pneumonitis when coupled with TBI.

All patients experienced severe nausea and vomiting as a consequence of chemotherapy and TBI. This lasted for about two weeks and gradually resolved.

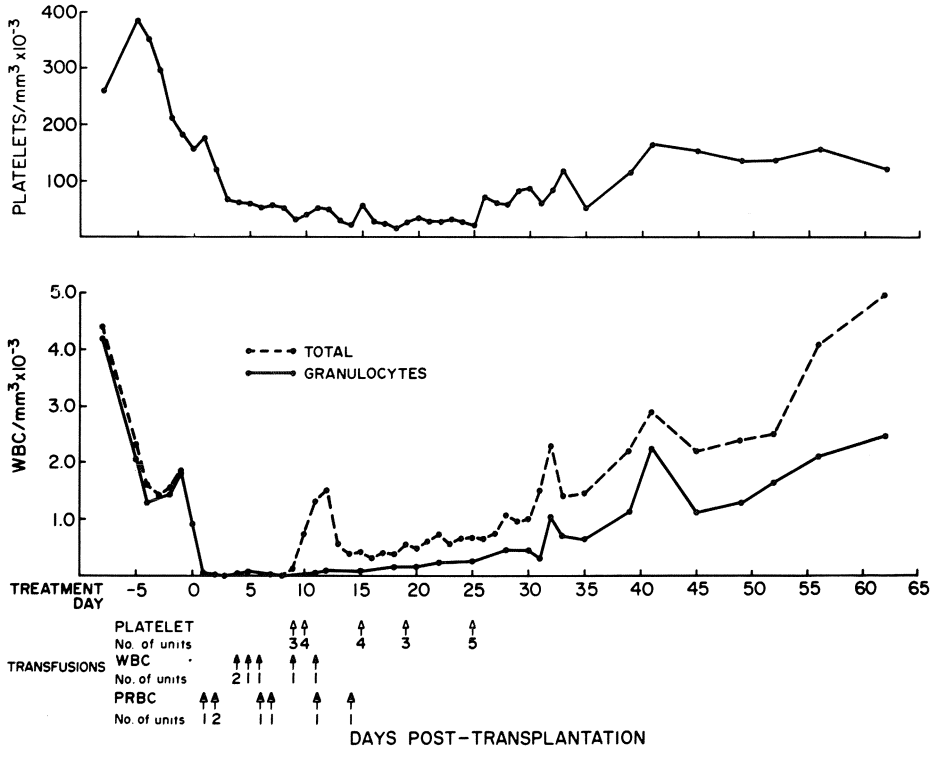


Fig. 1. Hematologic course of patient No. 1. Platelet, total white blood count and granulocyte count are shown with respect to time of marrow transplant (indicated as day 0). Time for transfusions of platelets, granulocytes (WBC) and packed red blood cells (PRBC) are shown along with the number of units given

Table 2. Summary of patients receiving autologous bone marrow transplants

Patient characteristics prior to autologous transplant therapy				Autologous marrow reinfused		Hematological recovery		Therapeutic response and follow-up data	
Patient No. and diagnosis	Age (yrs)/sex	Stage	Response to conventional therapy	Nucleated marrow cells per kg PT. weight	CFU-C per kg	Day WBC >1000	Day platelet >50,000	Antitumor response	Current status
1. Burkitt's lymphoma	6/M	IV	PR[a]	5×10^8	N.D.[b]	28	28	CR[c]	A + W[d] NED[e], 25 MOS.
2. Rhabdomyosarcoma	10/F	IV	Prog[f]	2.5×10^8	3.5×10^4	10	29	Prog	Dead day 49 tumor prog
3. Neuroblastoma	9/F	IV	PR	0.9×10^8	4.2×10^3	43	45	PR	A + W stable PR 16 MOS.
4. Neuroblastoma	5/M	IV	Prog	3.9×10^8	3.4×10^4	24	23	PR	Dead day 25 CMV infection
5. T-cell lymphoma	7/M	IV	Prog	1.1×10^8	4.4×10^3	33	–	Prog	Dead day 40 tumor Prog
6. Histiocytic lymphoma	27/F	IV	Prog	3.0×10^8	1.3×10^4	17	–	?CR	Dead day 73 pneumonitis
7. Small cell, lung	48/M	EXT	Per primum	1.5×10^8	1.4×10^4	14	26	Prog	Dead day 156 tumor Prog

[a] Partial response [b] Not done [c] Complete response [d] Alive and well [e] No evidence of disease [f] Progression of tumor

Despite the severe gastrointestinal toxicity, all patients maintained their weight with intravenous alimentation. Figure 1 shows the platelet count, total white blood count and granulocyte count of patient No. 1, plotted as a function of time of treatment, and is illustrative of the hematologic course of most patients. At about the time that patients receive their marrow reinfusion, they have become pancytopenic and aplastic. Many patients exhibit an early leukocyte peak, as is shown for patient No. 1, composed of mononuclear cells which morphologically resemble very young monocytes and lymphocytes. The significance of this early leukocyte peak is unknown, but it has also been observed in patients receiving allogeneic marrow transplants (unpublished observations). Leukocyte recovery exhibiting a more normal differential generally begins 2–4 weeks after transplant. The transfusional support shown on Fig. 1 is typical of that given to most patients. During the period of aplasia, all patients developed significant fever and were treated with broad spectrum antibiotics.

Despite the severity of post-transplant aplasia, only patients No. 4 and No. 6 exhibited irreversible, life threatening infection. Prior to transplantation, patient No. 4 had had intermittent fever and evidence suggestive of cytomegalovirus (CMV) infection. Because of this the transplant therapy had originally been deferred, but was begun when he exhibited evidence of rapid tumor progression. Shortly after transplant he manifested unusually severe diarrhea, hepatomegaly and interstitial pneumonitis with a rapid downhill course and death secondary to respiratory failure on day 25 post-transplant. Patient No. 6, despite adequate granulocyte recovery, remained intermittently febrile and 52 days post-transplant developed evidence of bilateral interstitial pneumonitis. Significantly, a blood buffy coat culture obtained three days prior to that time was positive for CMV. The pulmonary process progressed and the patient died 73 days after transplantation.

All patients achieved stable leukocyte counts above 1000 cells/mm^2 without transfusional support within six weeks of transplantation (Table 2). Except for patients No. 5 and No. 6, all achieved stable platelet counts exceeding 50,000/mm^3 within the same time frame. While the numbers are too small to be conclusive, there does appear to be a rough correlation between the nucleated marrow cell dose or CFU-C infused and the rate of hematologic recovery as shown on Table 2. The very rapid recovery of leukocytes in patient No. 2, however, was probably related in part to the fact that thoracic marrow was shielded during TBI.

The antitumor effects of therapy have been variable as summarized in Table 2. As previously indicated, two patients (No. 4 and No. 6) died as a consequence of therapy. At necropsy patient No. 4 showed evidence of marked tumor lysis but also had disseminated CMV infection of the bowel, liver and lung. Unfortunately, a post-mortem examination was not obtained on patient No. 6, so that the full extent of tumor regression could not be completely determined. Prior to the transplant she had had an abdominal recurrence which had been documented by surgery and excisional biopsy of an ovarian mass. A pretransplant computer axial tomographic (CAT) scan correlated well with the surgical findings of marked renal enlargment and massive periaortic lymphadenoapathy. About one month post-transplant a repeat CAT scan was normal. Patients No. 2 and No. 5 had brief

partial regressions of tumor but shortly thereafter developed tumor progression, which led to death on day 49 and 40 respectively. In patient No. 2, the initial tumor progression appeared in the right lung, and area shielded during TBI. In patient No. 5, the only patient to have had detectable prior marrow involvement, the initial relapse occurred in the bone marrow by day 28. Patient No. 7 had a partial regression of his primary tumor, but about two months later developed a cerebellar metastasis. Regional radiation slowed tumor growth but he died with progressive intrathoracic and intracranial disease at day 156. Two patients had significant therapeutic benefits from the procedure. In patient No. 3, the abdominal primary tumor decreased significantly in size and showed no evidence of growth in the first six months after treatment. An exploratory laporotomy carried out at that time revealed evidence of maturation of the neuroblastic cells in the tumor, but the mass remained unresectable. Further regression was achieved with regional irradiation and chemotherapy. Fourteen months post-transplant, a second laparotomy was successful at removing most of the residual mass with a small, still unresectable, mass of tumor being left at the celiac axis. She is alive and well, now over 16 months post-transplant, and is continuing chemotherapy and regional irradiation. On the basis of prior experience, this patient's expected survival after failing to achieve a complete remission would be 3 months or less. Patient No. 1 had a complete regression of tumor documented by a restaging laporotomy, five months post-transplant. His hemogram and marrow cellularity have remained normal since his recovery. He is now 25 months post-transplant and is probably cured.

II. In Vitro Antitumor Treatment of Marrow

Most cancers that are sensitive enough to cytotoxic therapy to potentially benefit from autologous marrow transplantation are either primary marrow malignancies, such as the acute leukemias, or have a high probability of having disseminated to the marrow by the time of diagnosis. As shown by the data on our clinical trials described above, about 10^{10} nucleated marrow cells are generally required for effective reconstitution of patients' hematologic function. Therefore, 0.1% tumor cells in the marrow, a level of contamination impossible to detect by current methods, would result in the reinfusion of 10^5 tumor cells. If autologous marrow transplantation is to have the widest applicability for the treatment of responsive cancers, some method for eliminating tumor cells from the marrow to be stored and subsequently reinfused is necessary.

Thierfelder and his colleagues have shown that the in-vitro treatment of normal AKR mouse marrow admixed with AKR leukemia cells with a rabbit anti-mouse thymocytic globulin (ATG) will eliminate clonogenic tumor cells [19]. The ATG used in these experiments had been exhaustively absorbed to reduce reactivity against normal AKR mouse cell surface antigenic determinants. We have described similar results in the 6C3HED mouse lymphoma model [6]. Table 3 summarizes the findings in this latter system. Lethally irradiated mice receiving 5×10^6 marrow cells plus 10^4 6C3HED tumor cells all died of tumor at about day 11 (group B). Treatment of this cell inoculum with either unabsorbed or absorbed rabbit anti-6C3HED anti-serum plus lytic complement resulted in close to 100%

Table 3. Summary of results of antiserum treatment of marrow plus 6E3HED tumor

Group	Intravenous inoculum			Inoculum pre-treatment	Survival of mice		
	No. lethally-irradiated mice	No. of C3H marrow cells	±No. of 6C3HED tumor cells	Antiserum ±complement	Fraction of survivors	Mean day of death ±SE	Cause of death
A	53	–	–	–	0/53	11.0±0.4	Marrow Failure
B	35	5×10^6	–	–	34/35	5	Infection
C	26	5×10^6	10^4	–	0/26	11.5±0.4	Tumor
D	16	5×10^6	10^4	U-R-a-6C[a]+C[b]	15/16	10	Infection
E	6	5×10^6	10^4	NRS[c]+C	0/6	11.2±0.2	Tumor
F	5	5×10^6	10^4	A-R-a-6C[d]+C	5/5	–	–

[a] Unabsorbed rabbit anti-6C3HED antiserum
[b] Lytic guinea pig complement
[c] Normal rabbit serum
[d] Absorbed rabbit anti-6C3HED antiserum

survival (groups D and F). Treatment of the cell inoculum with normal rabbit serum plus lytic complement resulted in all animals dying of tumor at the expected time (group E). Figure 2 shows that even the absorbed antiserum does not show absolute cytotoxic specificity for tumor cells since the dose of antiserum treated marrow cells required to rescue 100% of a group of lethally irradiated mice is always greater than that required for untreated marrow. The finding that relative cytotoxic specificity is all that is required for effective elimination of clonogenic tumor in the C3H model is of considerable significance since there is still a great deal of debate about the absolute specificity of tumor associated antigens [10].

Since both the AKR and C3H models are T-cell lymphoid malignancies, we have recently begun studies of a rat acute myelogenous leukemia (AML) model to demonstrate the broader applicability of this method for in vitro inactivation of tumor cells. Those properties which suggest that this model may be a reasonable analogue of human AML have been previously summarized by Vaughn et al. [25]. The mouse anti-LBN-AML antiserum we have thus far produced is, prior to absorption, cytotoxic to LBN-AML, normal LBN spleen and marrow cells and inhibits rat CFU-C at dilutions producing maximal cytotoxicity against LBN-AML cells. After absorbtion, the cytotoxicity to normal LBN spleen and marrow cells and inhibition of rat CFU-C is lost but cytotoxicity to LBN-AML cells is retained. The pattern of cytotoxic specificity against LBN-AML and normal LBM marrow cells, pre- and post-absorption, is correlated with findings with indirect IFA. Using the absorbed antiserum, we have begun studies similar to those summarized in Table 3 for the 6C3HED model. Thus far all animals to have received mixtures of 6.3×10^7 normal LBN marrow cells with 10^6 LBN-AML cells treated with control serum plus complement have died of tumor. While these experiments have been underway for only a short period of time, rats receiving normal marrow-tumor cell mixtures treated with absorbed mouse anti-LBN-AML plus lytic complement have at least a 50% increase in life span. If

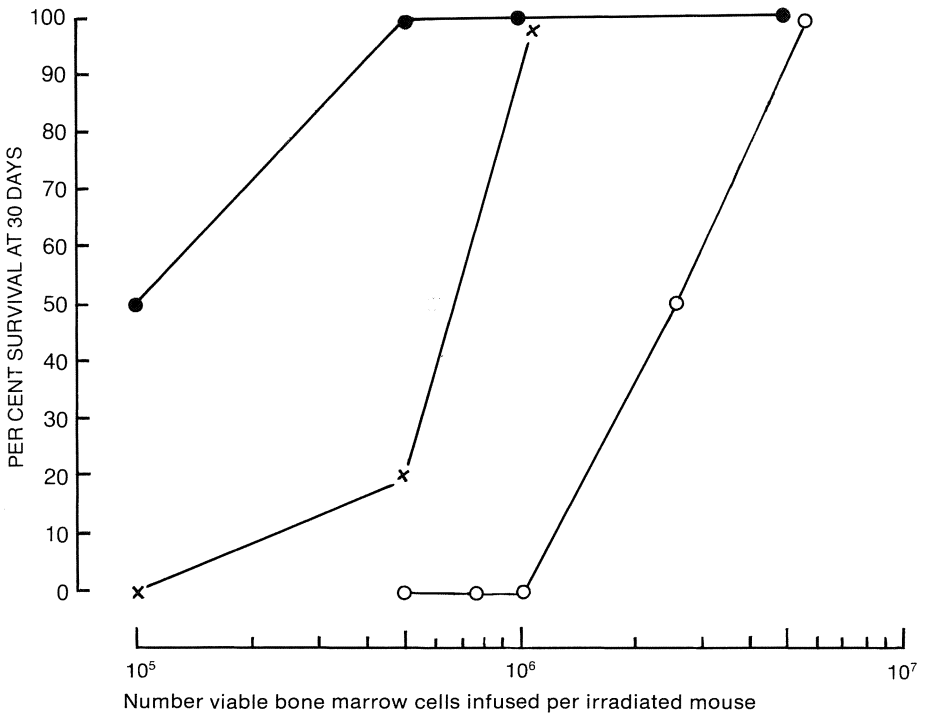


Fig. 2. Survival at 30 days of lethally irradiated C3H mice receiving varying numbers of untreated (closed circles), absorbed antiserum plus complement treated (crosses) or unabsorbed antiserum plus complement treated (open circles) C3H marrow. Each point represents four to ten mice

these animals continue to survive we will have duplicated our results in the 6C3HED model with rat AML and confirmed that this approach to eliminating clonogenic tumor contaminating marrow can be generalized to at least one non-lymphoid malignaney.

Whether these animal model studies can be generalized to apply to human autologous bone marrow transplantation remains to be seen. Certain precautions that must be taken, however, are illustrated by our experience with patient No. 5 which we have not previously described in detail. This 7 year old boy presented with a T-cell lymphoma which exhibited a leukemic conversion before he could be begun on therapy. Nonetheless, he reponded well to conventional remission induction therapy and we planned to store his complete remission marrow at the completion of consolidation therapy. Unfortunately, he relapsed before the completion of consolidation treatment. Although he remained responsive to subsequent cytotoxic therapy, we were never able to obtain a marrow containing less than 7–10% leukemic blasts. At about that time we learned of Thierfelder's work with the Ra-ATG which had properties resembling those of the ATG he had used in his AKR leukemia studies of in vitro antitumor treatment of marrow-tumor mixtures [14]. Dr. Thierfelder was kind enough to supply us with

some of this antiserum which we were able to show was cytotoxic to this patient's tumor cells under conditions which did not apparently inhibit the growth of CFU-C from his partial remission marrow. Because of the patients tenuous clinical condition, his marrow was harvested without further studies, at a time when it contained about 8% blasts. The marrow was treated with the ATG under the conditions which gave 100% cytotoxicity in the prior microtiter assay and then cryopreserved. The patient then received ADR, CTX, TBI and his cryopreserved treated marrow with initial complete disappearance of all evidence of tumor. Over the first three weeks after reinfusion of the treated marrow, his hematologic recovery progressed satisfactorily. Four weeks post-transplant, however, his marrow contained about 40% blasts and the patient progressed as described. It is not possible to determine if this patient's relapse resulted from the growth of tumor cells not destroyed by the pretransplant therapy or from tumor cells reinfused with the cryopreserved marrow. In this case, the rate of tumor progression allowed us little time for defining the optimum conditions for incubating the volumes of marrow required for clinical transplantation with antiserum and complement. We have carried out subsequent studies with Ho-ATG using absorbtion with Raji cells to reduce inhibitory activity against CFU-C. In the course of these studies we have compared cytotoxicity obtained by dye exclusion in the microtiter assay to cytotoxicity measured both by dye exclusion and ^{51}Cr release in an assay carried out with much larger volumes of incubation mixture. These studies have indicated that conditions providing 100% cytotoxicity in the microtiter plates do not yield maximal cytotoxicity when directly applied to larger incubation volumes.

The caveat that emerges from this clinical experience is that care must be taken when generalizing the results of any in vitro test of tumor cell inactivation to the conditions required for the clinical trial and scale-up experiments are an essential ingredient for success.

D. Summary and Conclusions

We have shown that it is possible to obtain and viably store sufficient numbers of stem cells to ensure durable hematological reconstitution of patients following marrow-lethal doses of chemoradiotherapy. While no current procedure can be guaranteed to eliminate clonogenic tumor from the bone marrow, the fact that hematopoietic stem cells capable of reconstituting the host can be obtained after intensive chemotherapy makes it possible to clear microscopic foci from the marrow prior to storage. Such patients are now included in our protocols. Animal model studies suggest that heterologous antitumor antisera of appropriate relative cytotoxic specificity may enable us to purge contaminated marrow of clonogenic tumor cells. Care, however, must be taken in defining the conditions of treatment for volumes of marrow required for clinical transplantation. Our initial treatment results indicate that in selected circumstances, tumor in otherwise refractory patients can be eliminated or partially controlled by a single intensive pulse of chemoradiotherapy, with severe, but acceptable extramedullary toxicity.

The fact that patients can be rescued from otherwise lethal myelotoxicity by reinfusion of cryopreserved autologous bone marrow permits wider exploration of new, more intensive cytoreductive regimens in a variety of cancers.

References

1. Abeloff, M. D., Ettinger, D. S., Khouri, N., et al.: Intensive induction therapy for small cell carcinoma of the lung. Cancer Treat. Rep., in press (1979)
2. Appelbaum, F. R., Herzig, G. P., Ziegler, I. L., et al.: Successful engrafting of cryopreserved autologous bone marrow in patients with malignant lymphoma. Blood *52*, 85–95 (1978)
3. Baker, M. A., Taub, R. N.: Production of antiserum in mice to human leukemia-associated antigens. Nature New Biol. *241*, 94 (1978)
4. Bond, V. P., Fliedner, T. M., Archambeau, J. O.: Mammalian radiation lethality: A disturbance in cellular kinetics. New York, London: Academic Press 1965
5. Deisseroth, A., Abrams, R. A.: The role of autologous stem cell reconstitution in the intensive therapy of resistant neoplasms. Cancer Treat. Rep., in press (1979)
6. Economou, J. S., Shin, H. S., Kaizer, H., et al.: Bone marrow transplantation in cancer therapy: Inactivation by antibody and complement of tumor cells in mouse syngeneic marrow transplants. Proc. Soc. Exp. Biol. Med. *158*, 449–453 (1978)
7. Goldin, A.: Factors pertaining to complete drug-induced remission of tumor in animals and man. Cancer Res. *29*, 2285–2291 (1969)
8. Graze, P. R., Gale, R. P.: Autotransplantation for leukemia and solid tumors. Transplant. Proc. *10*, 117–184 (1978)
9. Kaizer, H., Leventhal, B. G., Wharam, M. D., Munoz, L. L., Elfenbein, G. J., Tutschka, P. J., Santos, G. W.: Cryopreserved autologous bone marrow transplantation in the treatment of selected pediatric malignancies: A preliminary report. Transplant. Proc., in press (1979)
10. Leventhal, B. G., Weiner, M.: Leukemia antigens. In: The year in hematology. Silber, R., LoBue, J., Gordon, A. S. (eds.), pp. 463–479. New York: Plenum 1978
11. Lichter, A. S., Tutschka, P. J., Wharam, M. D., et al.: The use of fractionated radiotherapy as preparation for allogeneic bone marrow transplantation. Transplant. Proc., in press (1979)
12. Mann, D. L., Rogentine, G. N., Fahey, I. L., et al.: Solubilization and partial purification of human lymphoid cell surface antigens. J. Immunol. *103*, 282–292 (1969)
13. Northrup, J. E., et al.: Methods of in-vitro culture of granulocyte precursor cells, human and monkey. J. Natl. Cancer Inst. *48*, 629 (1972)
14. Rodt, H., Netzel, B., Niethammer, D., et al.: Specific absorbed antithymocyte globulin for incubation treatment in human marrow transplantation. Transplant. Proc. *9*, 187–191 (1977)
15. Rogentine, G. N., Yankee, R. A., Gart, J. J., et al.: HL-A antigens and disease: Acute lymphocytic leukemia. J. Clin. Invest. *51*, 2420–2428 (1972)
16. Santos, G. W.: Bone marrow transplantation. In: Advances in internal medicine, Vol. 24, pp. 157–182. Chicago, Ill.: Year Book Medical 1979
17. Santos, G. W. Sharkis, S. J.: Experience with syngeneic marrow transplantation in BN and WF rat models of acute myelogenous leukemia. In: Experimental hematology today. Baum, S. J., Ledney, G. D., (eds.), pp. 187–190. New York: Springer 1978
18. Santos, G. W., Tutschka, P. J., Elfenbein, G. J.: Marrow transplantation in acute leukemia following bulsufan and cyclophosphamide. In: Recent trends in the immunology of bone marrow transplantation. Thierfelder, S. et al. (eds.). Berlin, Heidelberg, New York: Springer 1980
19. Thierfelder, S., Rodt, H., Netzel, B.: Transplantation of syngeneic bone marrow incubated with leucocyte antibodies. I. Supression of lymphatic leukemia of syngeneic donor mice. Transplantation *23*, 459–463 (1977)
20. Thomas, E. D., Storb, R.: Technique for human marrow grafting. Blood *36*, 507–515 (1970)
21. Thomas, E. D., Storb, R., Clift, R. A. et al.: Bone marrow transplantation. N. Engl. J. Med. *292*, 832–843 (1975)
22. Tobias, J. S., Parker, L. M., Brown, B., et al.: Adriamycin and cyclophosphamide therapy for L1210 leukemia. Clinical Res. *23*, 344A (1975)

23. Tobias, J. S., Tattersall, M. N. H.: Perspectives in cancer research. Autologous marrow support and intensive chemotherapy in cancer patients. Eur. J. Cancer *12*, 1–8 (1976)
24. Bekkum, D. W. van, Oosterom, P. van, Dicke, K. A.: In-vitro colony formation of transplantable rat leukemias in comparison with human acute myeloid leukemia. Cancer Res. *36*, 941–946 (1976)
25. Vaughan, W. P., Burke, J. P., Jung, J.: BN rat myeloid leukemia transferred to the (LEW × BN)F_1 rat. J. Natl. Cancer Inst. *61*, 927–929 (1978)

Discussion

Bacigalupo: Greaves and coworkers have described the ALL-antigen as an example of tumor antigens first as tumor-specific. Now, they have found it in regenerating marrow, fetal tissues and fetal lymphocytes. How can you eliminate tumor cells with this antiserum?
Kaizer: It is inconceivable to me that antigens absolutely specific for tumors exist unless induced by viruses. All we need is a relative specificity. In the C_3H system we have only one log difference of the absorbed antiserum for tumor cells vs stem cells and we got rid of the tumor cells.
Kersey: I would like to comment to your success which is reminiscent of a case of Burkitt-lymphoma in Minneapolis who had been treated with high dose chemotherapy, total body irradiation and an allogeneic marrow graft three years ago and is now in complete, unmaintained remission. A second patient treated with autologous marrow died of venoocclusive liver disease after three months. At autopsy there was no tumor. Burkitts lymphoma is an interesting tumor for such studies and one should do cooperative studies with it.
Kaizer: With the incidence of Burkitts lymphoma the suggestion of cooperative studies is a reasonable one, but there are other tumors as well.
Fliedner: One of the first tasks of autologous bone marrow transplantation would be to find not minimum number, but the optimum number of stem cells necessary for rapid recovery. I was wondering about your low recovery after preservation, we find a 90–95% recovery of CFU-C after cryopreservation. Is your reference number that of total nucleated marrow cells including granulocytes instead of mononuclear cells? Have you followed CFU-C levels in marrow and blood after transplantation? Have you tried to separate tumor cells from the marrow by physical means?
Kaizer: We use as a base total nucleated cells, we do not separate mononuclear cells and sometimes we get clumping. This may, account for our low recovery. We have not followed up enough CFU-C after transplantation to report on. I have not felt that physical means can help to get rid of the last clonogenic tumor cell.
Prentice: We have used yet another way of eliminating tumor cells by positively selecting stem cells. Stem cells carry ALL-antigens, Ia-antigens and the TdT enzyme. In the T-cell leukemia it may be logical to select Ia-positive stem cells from the bone marrow. Using this approach we have already demonstrated that we can get full CFU-C recovery. In contrast the ALL antigen is too weak for this approach.
Dupont: I would object to your statement that you need viral DNA for tumor antigens, you could find derepression. The melanoma antigen is an example of tumor specific antigen. The presence of Ia-antigens on marrow stem cells may also be a problem, since 50 per cent of melonoma cells express Ia antigens.
Kaizer: All I am saying is that one needs a relative specificity and in the end a combination of these methods will be used to eliminate tumor cells.

The Concept of Antileukemic, Autologous Bone Marrow Transplantation in Acute Lymphoblastic Leukemia*

B. Netzel, H. Rodt, R. J. Haas, G. Janka, and S. Thierfelder

A. Introduction

The lack of histocompatible bone marrow donors has hitherto set a strong limitation on the treatment of advanced stages of acute leukemia by bone marrow transplantation, where cytoreductive agents and total body irradiation have to be used in supralethal doses.

In our opinion the rescue of normal hemopoiesis is possible by use of autologous marrow which has been collected during the remission phase of the disease and stored at low temperatures.

Bone marrow removed from leukemic patients during remission may contain residual leukemic cells and therefore has to be freed of them prior to autotransplantation.

Various efforts have been made to eliminate in vitro leukemic cells from normal marrow cells, using discontinuous gradient techniques [1] or by means of specific antibodies against leukemic cell surface determinants [5, 6, 7].

Recently, heterologous antisera were prepared against cALL and T antigens [10, 11]. These antisera show high cytotoxic activity against leukemic cells of cALL and T-ALL, both subgroups representing more than 90% of childhood ALL [7].

Our studies provide evidence that these antisera reveal specific toxicity against the target cells without interfering with normal hemopoietic stem cells [8, 6]. The nonreactivity of the antisera against stem cells allows us to use them therapeutically in autologous marrow transplantation.

In previous studies on mice, we could already demonstrate that rabbit antiserum against mouse T cells killed over 99% of cells when incubated with a T cell AKR/J leukemia transferred subsequently to syngeneic hosts.

While recipients conditioned with 800 R and given syngeneic bone marrow mixed with leukemic cells died within 11 days from leukemia, no leukemia was detected during the observation period of 100 days if the leukemic bone marrow had been preincubated with anti-T cell globulin and complement [14].

Another critical aspect in autologous bone marrow transplantation is the evaluation of stem cell potential during the remission phase of the disease. Significant differences in stem cell potential have been found in our patients undergoing various chemotherapy protocols. The analysis of the data gives us the

* Supported in part by Deutsche Forschungsgemeinschaft, SFB 37, E 9 and Deutsche Krebshilfe, Bonn, Tumorzentrum München

rationale for the optimal time of bone marrow collection during the remission phase.

Finally, new techniques for processing of bulk marrow collected for autotransplantation have been introduced. This includes the use of a cell separator for concentration of mononuclear cells in a small volume and reinfusion of donors erythrocytes [9].

Autologous transplantation requires the facilities of a bone marrow bank with long-term storage of marrow cells in liquid nitrogen for several years until the patient has a leukemic relapse. Monitoring of viability of cryopreserved bone marrow after various storage intervals revealed that marrow stem cells can be preserved in liquid nitrogen for several years without significant loss of stem cell function [9], and cryopreserved marrow cells have recently been successfully applied to autologous bone marrow transplantation [1, 2, 4, 13].

B. Patients, Materials and Methods

Patients at the University Children's Hospital Munich were treated according to the chemotherapy protocol 75/76, using vincristine and prednisone in the induction phase and prophylactic cranial irradiation within the first 10 weeks. Patients on a later protocol (77–01) received vincristine, prednisone, asparaginase and adriamycin for induction of remission, followed by methotrexate, cyclophosphamide, cytosin-arabinoside, vincristine and prednisone to effect early consolidation. Central nervous system prophylaxis comprises 2400 rad cranial irradiation using a ^{60}Co unit, and intrathecal administration of methotrexate. Treatment in remission in both protocols involves 6-mercaptopurine daily and methotrexate weekly. Therapy is discontinued after 30 months [3].

Bone marrow and blood samples from patients with ALL were obtained from routine punctures at diagnosis and at three-month intervals during chemotherapy and after cessation of maintenance therapy and assayed for CFU-C.

I. Cell Preparations

Normal mononuclear cells and ALL cells were separated from peripheral blood and bone marrow by Ficoll-Isopaque density sedimentation. ALL blasts, used for the production of antisera, were obtained from untreated patients at the University Children's Hospital, Munich.

II. Antisera

Antisera against human thymocytes and lymphoblasts of patients with common acute lymphoblastic leukemia were developed in rabbits. Immunization, absorption and purification procedures are described in detail elsewhere [10, 11].

III. Colony Inhibition and CFU-C Assay

Cross-reactions of the antisera with hemopoietic stem cells and progenitors were measured culturing bone marrow cells after incubation of absorbed and nonabsorbed antibody preparations for 30 min at 4° C. Selected normal rabbit serum was added as the source of complement and the suspension was further incubated for 60 min at 37° C. Thereafter the cells were washed, counted and cultured in the CFU-C assay. The number of stem cells committed to the granulocyte-monocyte series (CFU-C) was evaluated by a double-layer agar technique and is described in detail [8].

IV. Complement Fixation Test

Complement fixation with cells as antigens was used as a quantitative micromethod [10]. The 50 percent lysis of sheep red cells was defined as the titer of the antiserum when the number of cells was kept constant.

V. Cell Separator

Remission marrow collected for autotransplantation was aspirated under general anesthesia from the anterior and posterior iliac crest [15] and suspended in preservative-free heparin-TC 199 medium without phenolred.

The marrow was processed using a semi-continuous flow cell separator (Haemonetics Model 30) equipped with a 100 ml centrifuge bowl. The buffy coat was collected in 2–4 cycles over a 40–55 sec period at a filling rate of 40 ml per min and a collection rate of 20 ml per min. The red cell suspension, depleted of mononuclear cells, was washed twice and immediately reinfused to the donor.

VI. Cryopreservation

Cell suspensions used as test samples were diluted at 4° C with an equal volume of 20% DMSO and 40% AB serum in TC 199 medium and filled into 2 ml plastic vials.

Freezing was performed in a CRF-2 freezing chamber at a rate of 1° C per min to −30° C, than at a rate of 5–7° C per min to −100° C and then stored in liquid nitrogen. Thawing was performed rapidly within 2–3 min. The cell suspension was diluted stepwise (15 min) with 10 volumes of 20% serum in TC 199 medium, centrifuged and then subjected to total and differential counts.

Marrow cells used for autotransplantation were transferred to 100 or 200 ml UCAR bags and mixed with freezing medium at 4° C to a final concentration of 10% DMSO, 20% autologous plasma and sandwiched in aluminium sleeves. The freezing process was controlled by placing the thermocouple in a test bag sandwiched in the same way, and freezing was performed as described.

C. Results and Conclusions

I. Activity of Heterologous Antibodies on Leukemic Cells and Normal Hemopoietic Cells

A short term incubation of marrow cells with nonabsorbed rabbit antithymocyte globulin or rabbit anti-cALL globulin and complement completely inhibited the clonal growth of stem cells committed to the granulocyte-monocyte series (Fig. 1, 2) and led to a loss of proliferation and differentiation in diffusion chambers [6].

The crude antisera contain not only specific antibodies against T antigen or cALL antigen but also antibodies against a variety of different antigenic determinants (species-specific, cell-specific) present on cells other than T-cells or cALL-cells. These antibodies with specificities directed against antigens shared by cells of different origin are in part responsible for the inactivation of normal hemopoietic stem cells.

The stepwise removal of cross-reacting antibodies by absorption procedures led to increasing specifity of the antisera and increasing survival of hemopoietic precursor cells.

When marrow cells were incubated with antithymocyte globulin absorbed with liver-kidney homogenate, pooled B-cells from chronic lymphatic leukemias or ATG absorbed additionally with a pool of B-type lymphoblastoid cell lines, no inhibitory activity against CFU-C could be detected (Fig. 1), whereas high cytotoxic activity against T cells was preserved [10].

Absorption of anti-cALL with liver-kidney homogenate, cells from chronic lymphatic leukemias and lymphoblastoid cells from B cell lines removed the cytotoxic effect of anti-cALL against CFU-C completely (Fig. 2) and did not inhibit proliferation of marrow cells and CFU-C in diffusion chambers [6], while

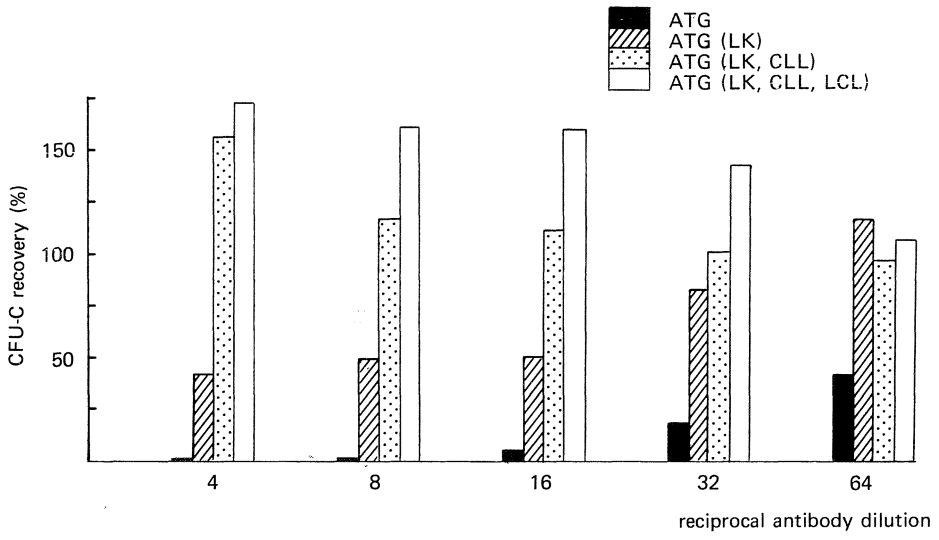


Fig. 1. Recovery of CFU-C after incubation of marrow cells with enhancing dilutions of crude and differently absorbed ATG in the presence of complement. 100%=control value (marrow cells incubated with normal rabbit globulin in appropriate dilutions in the presence of complement). ATG (LK)=absorbed with liver-kidney homogenate, ATG (LK, CLL)=absorbed additionally with pooled B-cells from patients with chronic lymphatic leukemias, ATG (LK, CLL, LCL)=absorbed additionally with a pool of B-type lymphoblastoid cell lines. All preparations were adjusted to the same cytotoxic activity against T-cells using the complement fixation test for determination of activity

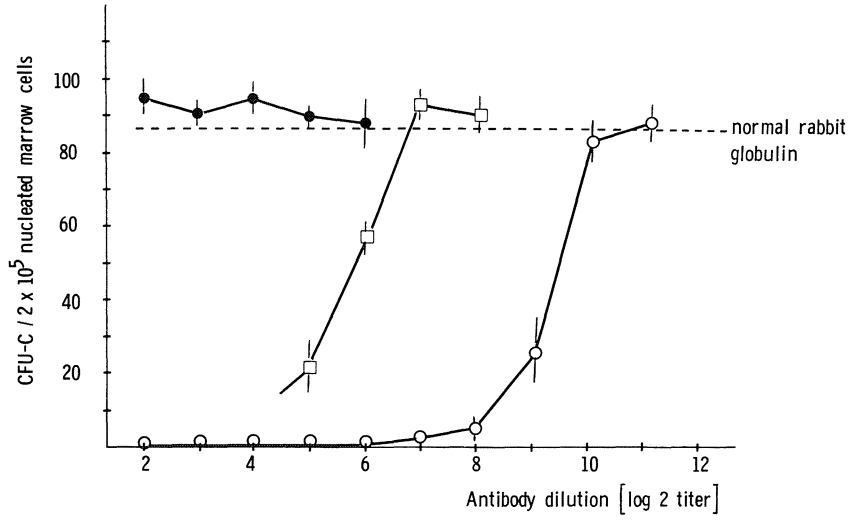


Fig. 2. Recovery of CFU-C after incubation of normal marrow cells with increasing dilutions of differently absorbed anti-cALL and complement. ○, anti-cAll not absorbed; □, absorbed with red blood cells and CLL; ●, absorbed with red blood cells, CLL, and LCL

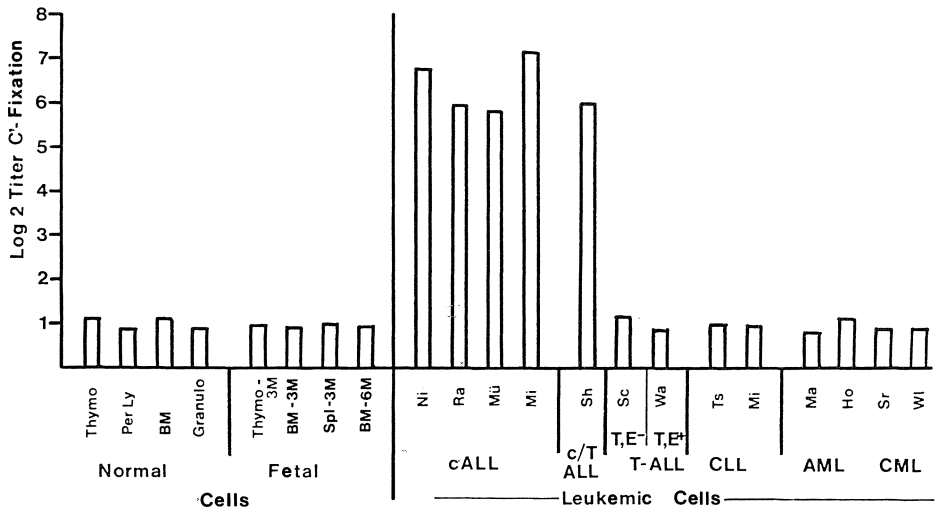


Fig. 3. Reactivity of anti-cALL globulin against various normal, fetal and leukemic cell populations in the complement fixation test

high cytotoxicity against cALL blasts was still present [6]. The specific activity of absorbed anti-cALL is illustrated in Fig. 3. The antiserum did not react with various normal and fetal cell populations in the complement fixation test, such as thymocytes, peripheral blood lymphocytes, bone marrow cells and granulocytes. No reaction was observed with fetal cells of the thymus, spleen and bone marrow in months 3 and 6. The reactivity of anti-cALL against T-ALL, chronic lymphatic leukemia, acute or chronic myeloid leukemia was also negative, whereas a strong positive reaction was observed with cALL as well as with intermediary leukemias marked both by T-antigen and cALL-antigen [12].

Our in vitro studies provide evidence of the lack of an inhibitory effect of anti-T and anti-cALL globulin on normal hemopoietic stem cells. The nonreactivity of these antisera against stem cells enables us to use them for the elimination of residual leukemic cells in the bone marrow taken during remission and stored for retransplantation in relapse.

II. Stem Cell Potential in ALL Patients During the Course of the Disease

Apart from the problem of early relapse in ALL, autologous marrow transplantation may be limited by the transplantation potential of marrow from patients undergoing intensive induction chemotherapy and maintenance chemotherapy for a period of 30 months.

The stem cell potential of bone marrow and blood samples from patients before chemotherapy and during the remission phase of ALL has been investigated in two collectives treated with different cytoreductive regimens.

Figure 4 summarizes the distribution patterns of CFU-C in 160 children with ALL at diagnosis and during maintenance chemotherapy.

At the time of diagnosis of ALL no or very low levels of CFU-C were found in the bone marrow aspirates from 68 patients compared to a median CFU-C value of 6295/ml obtained from 157 samples of remission bone marrow and compared to normal marrow (Fig. 4).

In contrast, in the peripheral blood from 79 patients 200 times more CFU-C/ml blood were found at diagnosis compared to remission values (2666 CFU-C/ml versus 11 CFU-C/ml, mean).

At diagnosis, the great majority of total CFU-C are present in circulation and not in the bone marrow, which is overcrowded by leukemic cells. From our data we can estimate that the total circulatory pool of stem cells before chemotherapy is greater than the total pool of stem cells in remission bone marrow exposed to continuous chemotherapy. Therefore one may speculate on collecting stem cells for autotransplantation from peripheral blood before they are reduced by chemotherapy, at least in such patients who have only few leukemic cells in circulation and provided that appropriate antisera are used to eradicate residual leukemic cells.

During maintenance chemotherapy, statistically significant lower CFU-C per ml blood were observed compared to healthy children (11 CFU-C/ml versus 131 CFU-C/ml mean, $p<0.001$). During that period it is obviously impossible to collect from peripheral blood sufficient amounts of stem cells for autotransplantation.

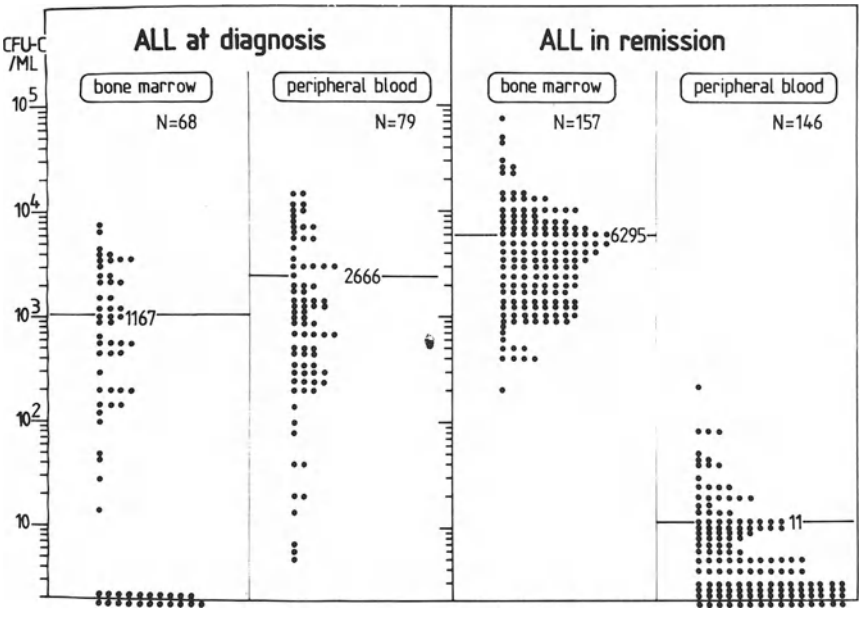


Fig. 4. CFU-C data from ALL patients at diagnosis and in remission. 10^5 and 2×10^5 mononuclear cells were plated. Each point represents the mean of at least three culture dishes. Control values from normal bone marrow; 15712 ± 10133 CFU-C/ml bone marrow ($n=24$)

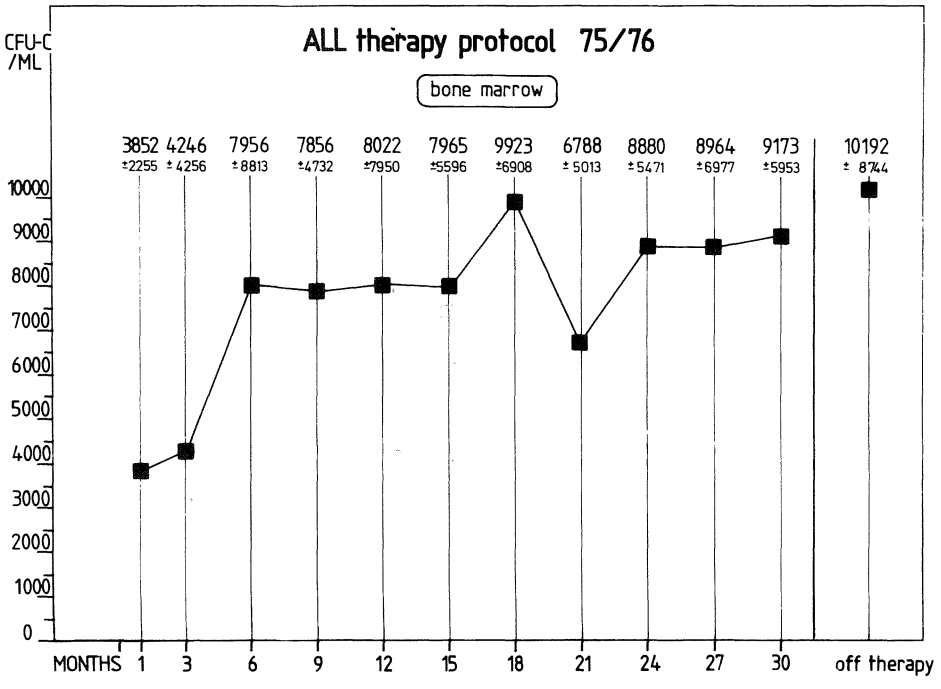


Fig. 5. CFU-C from patients with ALL in remission, treated according to the therapy protocol 75/76

Monitoring of CFU-C in bone marrow was performed during maintenance chemotherapy in two groups of patients treated according to different chemotherapy protocols.

Figure 5 summarizes data from patients in continuous remission, treated according to the protocol 75/76 (see methods), on whom a check was kept for up to 28 months after the maintenance chemotherapy had been concluded. Interestingly, no significance in CFU-C/ml bone marrow was observed between patients off therapy and patients in whom remission bone marrow was collected 6 months or later after the beginning of chemotherapy. In contrast, significant lower CFU-C/ml bone marrow was observed 1 and 3 months after induction therapy. From this group of patients we collected bulk marrow for autotransplantation not earlier than 6 months after the beginning of chemotherapy.

Data from patients who were treated according to the chemotherapy protocol 77–01 (see methods) were summarized in Figure 6. It is clearly demonstrated that this regimen is more toxic for stem cells, and during the first 3 months we could observe significant differences in CFU-C/ml marrow between the two protocols and a delayed regeneration of the bone marrow stem cell pool in the former patient group. These data imply that bone marrow should be collected in a later remission phase, although this may be a misleading conclusion because the risk of a relapse increases.

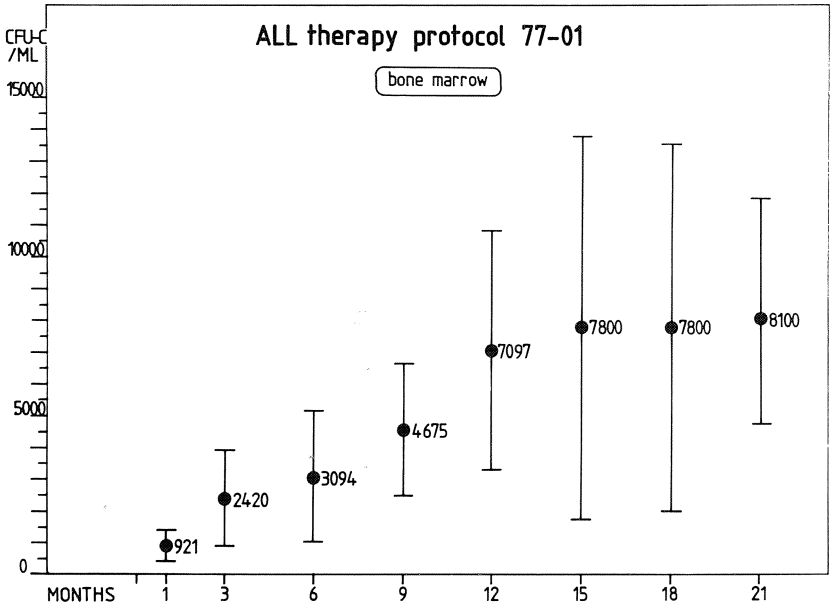


Fig. 6. Bone marrow CFU-C from patients, treated according to the therapy protocol 77–01

III. Processing of Marrow Cells by a Semi-continuous Flow Cell Separator

Bone marrow cells collected for autotransplantation from children with ALL in remission were separated by a cell separator (Haemonetics Model 30) equipped with a 100 ml centrifuge bowl. This procedure resulted in a concentration of mononuclear cells and stem cells in a small volume and allowed freezing and storage of marrow cells in 100 to 200 ml bags. In addition, up to 97% of donor erythrocytes could be retransfused to the patient immediately after pheresis.

Table 1 summarizes data from 29 patients with ALL in remission, from whom marrow has been processed by the cell separator.

IV. Bone Marrow Bank

Autologous marrow transplantation as an integral part of therapy in relapsed acute lymphoblastoid leukemia requires the facilities of a bone marrow bank with

Table 1. Processing of bone marrow cells by the semi-continuous flow cell separator

	Initial volume (ml)	Volume after pheresis (ml)	Total yield of MNC (%)	Total yield of CFU-C (%)[a]
Mean (N=29)	928	211	86	89
Range	625–1215	102–330	76–94	78–95

[a] CFU-C/10^5 and 2×10^5 mononuclear cells, plated in triplicates before and after pheresis

long term storage of marrow cells in liquid nitrogen for several years. In our center remission bone marrow from 34 patients has been stored during the past 2 years.

The survival of stem cells during prolonged storage intervals has been investigated in marrow samples from 92 healthy persons and from 50 patients with ALL in remission. Samples were stored in liquid nitrogen for periods up to 5½ years and assayed for CFU-C after various storage intervals (Table 2, 3).

Table 2. Recovery of CFU-C from normal bone marrow cells

	1 year	1–2 years	2–4 years
CFU-C[a] recovery %	84.2 (n=92)	81.7 (n=42)	79.1 (n=31)
Total MNC[b] recovery %	77±22 range 34–114 (n=68)	n.t.	73±18 range 41–122 (n=23)

[a] CFU-C/10^5 and 2×10^5 mononuclear cells, plated in triplicates before and after pheresis
[b] After washing and removal of DMSO, mean±SEM

Table 3. Recovery of CFU-C from remission bone marrow cells

	1 year	1–2 years
CFU-C[a] recovery %	86.4 (n=81)	83.3 (n=32)
Total MNC[b] recovery %	79±16 range 65–109 (n=42)	81±26 range 54–131 (n=23)

[a] All marrow samples were plated per 10^5 and 2×10^5 mononuclear cells (MNC) for CFU-C prior to freezing (=100% value)
[b] After washing and removal of DMSO, mean±SEM

No significant difference in recovery of CFU-C between marrow cells from normal persons and children with ALL in remission could be observed. So far, the transplantation potential of bone marrow stem cells exposed to maintenance chemotherapy in ALL patients was not affected during the process of cryopreservation.

Recovery of CFU-C did not differ significantly with various storage intervals (<1 year, 1–2 years, 2–4 years), some of them have been followed for up to 5½ years.

The total MNC recovery after thawing was 100% (mean), removal of DMSO led to a loss of MNC (73–81% recovery) most probably due to cell clumping during the washing procedure.

This loss of marrow cells has to be taken in account during collection of marrow cells for autotransplantation.

Acknowledgement

We would like to thank Ursula Scherello, Hermine Manz und Susanne Trimborn for the expert technical assistance.

References

1. Dicke, K. A., McCredie, K. B., Spitzer, G., Zander, A., Peters, L., Verma, D. S., Stewart, D., Keating, M., Stevens, E. E.: Autologous bone marrow transplantation in the patients with adult acute leukemia in relapse. Transplantation *26,* 169 (1978)
2. Dicke, K. A., Zander, A. Spitzer, G., Verma, D. S., Peters, L., Vellekoop, L., McCredie, K. B., Hester, J.: Autologous bone-marrow transplantation in relapsed adult acute leukemia. Lancet Vol. *1,* 8115 (1979)
3. Haas, R. J., Netzel, B., Janka, G. E., Rodt, H., Thiel, E., Thierfelder, S.: Diagnostischer Einsatz spezifischer Antiseren bei der akuten lymphatischen Leukämie im Kindesalter. Klin. Pädiatr. *191,* 446 (1978)
4. Kaizer, H., Wharam, M. D., Johnson, R. J., Economou, J. S., Shin, H. S., Santos, G. W., Elfenbein, G. J., Leventhal, B. G.: Requirements for the successful application of autologous bone marrow transplantation in the treatment of selected malignancies. In: Recent trends in the immunobiology of bone marrow transplantation. Thierfelder, S. et al. (eds.). Berlin, Heidelberg, New York: Springer 1980
5. Netzel, B., Haas, R. J., Rodt, H., Thiel, E., Thierfelder, S.: Antisera against human leukemia-associated antigens for treatment of childhood acute lymphoblastic leukemia. In: Immunotherapy of malignant diseases, p. 332. Stuttgart, New York: Schattauer (1978)
6. Netzel, B., Rodt, H., Lau, B., Thiel, E., Haas, R. J., Dörmer, P., Thierfelder, S.: Transplantation of syngeneic bone marrow incubated with leukocyte antibodies. II. Cytotoxic activity of anti-cALL globulin on leukemic cells and normal precursor cells in man. Transplantation *26,* 157 (1978)
7. Netzel, B., Rodt, H., Thiel, E., Haas, R. J., Tierfelder, S.: Recent aspects on the use of specific antisera in diagnosis and therapy of acute lymphatic leukaemia in infants. Klin. Pädiatr. *191,* (1979)
8. Netzel, B., Rodt, H., Hoffmann-Fezer, G., Thiel, E., Thierfelder, S.: The effect of crude and differently absorbed anti-human T-cell globulin on granulocytic and erythropoietic colony formation. Exp. Hematol. *6,* 410 (1978)
9. Netzel, B., Haas, R. J., Janka, G. E., Thierfelder, S.: Viability of stem cells (CFU-C) after long termin cryopreservation of bone marrow cells from normal adults and children with acute lymphoblastic leukemia in remission. In: Cell separation and cryobiology. Rainer, H., Borberg, H., Mishler, J. M., Schäfer, U. (eds.), p. 255. Stuttgart, New York: Schattauer 1978
10. Rodt, H., Thierfelder, S., Thiel, E., Götze, D., Netzel, B., Huhn, D., Eulitz, M.: Identification and quantitation of human T cell antigen by antisera purified from antibodies cross-reacting with hemopoietic progenitors and other blood cells. Immunogenetics *2,* 411 (1975)
11. Rodt, H., Netzel, B., Thiel, E., Jäger, G., Huhn, D., Haas, R. J., Götze, D., Thierfelder, S.: Classification of leukemic cells with T and O-ALL specific antisera. In: Haematology and blood transfusion, Vol. 20, p. 87: Immunological diagnosis of leukemias and lymphomas. Thierfelder. S., Rodt, H., Thiel, E. (eds.). Berlin, Heidelberg, New York: Springer 1977
12. Rodt, H., Netzel, B., Thiel, E., Thierfelder, S.: Classification of acute lymphoblastic leukemias by antisera directed against ALL-associated antigens. In: Proc. Intern. Sympos. on Therapy of Acute Leukemias. Mandelli, F. (ed.). Rom: Lombardo 1979
13. Schaefer, U. W., Dicke, K. A., Bekkum, D. W. van: Recovery of haemopoiesis in lethally irradiated monkeys by frozen allogeneic bone marrow grafts, Rev. Europ. d'Etudes Clin. Biol. *17,* 483 (1972)
14. Thierfelder, S., Rodt, H., Netzel, B.: Transplantation of syngeneic bone marrow incubated with leukocyte antibodies. I. Suppression of lymphatic leukemia of syngeneic donor mice. Transplantation *23,* 460 (1977)
15. Thomas, E. D., Storb, R.: Technique for human marrow grafting. Blood *36,* 507 (1970)

Discussion

Fliedner: CFU-C is, as we all know, a good start for testing stem cell content of human marrow. Have you tested other stem cell tests like BFU-E for example?
Netzel: We have preliminary results on BFU-E and CFU-E.
Fliedner: Only the parallel behaviour of more than one test tells us something about the pluripotent stem cell.
Dicke: Did you do morphology before and after freezing on yours colonies? Did you see differences?
Netzel: We saw no important differences, sometimes we saw eosinophilic colonies.
Dicke: We saw great differences, for instance many eosinophilic colonies after thawing. I agree to Dr. Fliedner that you need additional assays like the BFU-E assay.
Prindull: We found a poor correlation between CFU-C in bone marrow and in blood of children with ALL.
Netzel: We have never found low CFU-C levels in blood before therapy.
Simonsen: Wouldn't it be possible to exploit the fluorescent cell sorter for counting of leukemic contaminants?
Netzel: I have exploited the cell sorter at the DKFZ/Heidelberg with this technique and the problems lie in the high background which at least is about 1 per cent.
Thierfelder: From the mouse model we know that we are eliminating more than 99,9 per cent of T-leukemia cells by incubating the marrow with antiserum. The background of the cell sorter is more than one per cent.
Kersey: I was fascinated by your demonstration of slow recovery of CFU-C for more than 1 year, we have studied immune recovery and found it to be slow. Have you studied recovery of immune function?
Eife: The data are on their way.
Prentice: I want to emphasize that the ALL antigen is a normal antigen present on the pluripotential stem cells. We have seen up to 20 percent ALL positive cell in the bone marrow post engraftment, in a child with aplasia up to 5 per cent and in several paticuts in a rapid recovery phase post chemotherapy. I think that your patients given the anti-ALL serum incubated marrow will get grafts, but these grafts will fade out like in Dr. Schäfer's patient because you get grafts of committed stem cells without pluripotential stem cells. We know that this ALL-antigen is present on pluripotential stem cells.
Netzel: In some cases we have found 20 per cent unspecific fluorescent positive cells in the marrow, but we are unable to conclude that these represent 20 per cent pluripotent stem cells. Nevertheless, we are concerned that none of the vitro tests are predictive, but we have been unable to demonstrate cytotoxic activity of this serum against cells growing in diffusion chambers and the combination of diffusion chamber and CFU-C assay.
Kaizer: I would like again to emphasize that one needs only relative specificity to clear a marrow of clonogenic tumor cells and this is the experiment proposed here which is crying to be done.

Autologous Bone Marrow Transplantation in Relapsed Adult Acute Leukemia*

K. A. Dicke, A. R. Zander, G. Spitzer, D. S. Verma, L. Peters, L. Vellekoop, S. Thomson, D. Stewart, and K. B. McCredie

A. Summary

From March, 1976 to February, 1979, 28 cases of adult acute leukemia of which 24 were evaluable were treated in irreversible relapse with high dose chemotherapy (piperazinedione) and supra-lethal total body irradiation (TBI) in conjunction with autologous bone marrow transplantation (ABMT). The marrow cells grafted were collected and stored in liquid nitrogen at the time of remission. In 12 patients the marrow cells were fractionated using discontinuous albumin gradients in an attempt to separate normal cells from residual leukemic cells. Twelve patients achieved complete remission (CR); in 9 additional patients signs of engraftment were evident but death occurred before achievement of CR. Seven of 12 AML patients, which were treated with bone marrow transplantation as first treatment of their relapse, achieved CR. Four of 5 patients with ALL, whose bone marrows were collected during first remission, reached CR. The median CR duration was 4+ months and the median survival of the patients reaching CR was 6+ months. Autologous bone marrow transplantation offers a good chance of CR (66%), when marrow is collected during first remission and used as first treatment for AML in third relapse and ALL in second relapse.

B. Introduction

Recent advances in the treatment of adult acute leukemia have led to improved CR rates and survival duration [10, 11]. However, despite this progress the majority of adult acute leukemia patients die of recurrence of leukemia within two years after onset of the disease.

The prognosis of adult acute myeloblastic leukemia (AML) after the first relapse is dismal. Only 30% achieve a second remission of short duration with an overall survival of 18 weeks [2]. The survival of patients with acute lymphoblastic leukemia (ALL) and undifferentiated leukemia (AUL) in their second relapse equals that of patients with AML in their first relapse [16]. Efforts to overcome the resistance of relapsed leukemia with high dose chemotherapy plus TBI followed by allogeneic bone marrow transplantation from HLA-identical, MLC-negative donors have been proven successful. However, allogeneic bone marrow transplantation is limited by the scarcity of suitable donors – only 30% of

* Supported by grant Nos. CA-23077, CA-14528, CA-5831 from the NIH, Bethesda, Maryland

our leukemia patients have HLA-identical donors. There is a large early mortality up to 75% within three months after transplantation due to infectious complications, interstitial pneumonia and graft versus host (GvH) disease as a result from immune incompatibility. Only up to 17% of the transplanted patients survive two years after transplantation in CR [25, 27]. Results generated by the Seattle group in syngeneic bone marrow transplantation i.e., transplantation of marrow from identical twins, showed better long term survival due to absence of fatal GvH disease and decreased incidence of interstitial pneumonia [9]. Syngeneic bone marrow transplantation is limited by the very low incidence of identical twins.

As an alternative to the syngeneic and allogeneic bone marrow transplantation, we have developed an ABMT program for relapse leukemia utilizing the patient's own cryopreserved remission bone marrow for hemopoietic reconstitution after high dose chemotherapy and irradiation [5]. Our study reports 8 patients transplanted with autologous remission bone marrow following high dose chemotherapy and TBI. In an attempt to reduce possible leukemia cell contamination of the marrow cell suspension collected during remission, we introduced physical separation by discontinuous albumin density gradient centrifugation of the cells in 12 patients.

C. Patients and Methods

I. Patient Group

Seventeen patients had acute myeloblastic leukemia, 9 acute lymphoblastic and 2 acute undifferentiated leukemia. The median age was 28 with a range from 18 to 48 years. All except 5 bone marrows were aspirated during first remission. Patients with ALL and AUL entered the transplantation program in second, third and fourth relapse; patients with AML in first and second relapse. All patients had received extensive induction, consolidation, maintenance and late intensification therapy [10]. Fifteen patients had received second line chemotherapy, two even investigational chemotherapy and were considered failures. Twelve patients entered the transplantation program without receiving second line chemotherapy. In three patients, (2 AML, 1 ALL) the toxicity of the program, the anti-leukemic effect of the cytoreductive regimen and the hemopoietic capacity of the infused marrow could not be evaluated because of early death (within 5 days after transplant) due to preexisting conditions as fungemia, liver failure and pseudomonas septicemia. Therefore, 21 patients are evaluable. In another patient (ALL) the effect of piperazinedione and TBI could not be evaluated since additional drugs, cytoxan and VP-16 were administered. For this reason this patient was ecluded from analysis.

Twelve of the 28 patients, 10 of the 24 evaluable cases received fractionated marrow. The decision of marrow fractionated before storage depended on the work load of the laboratory, namely, on the number of other marrows simultaneously being frozen, an important determinant in quality control of freezing and thawing procedures.

II. Preparation of Bone Marrow Suspensions

Cells were collected by multiple bone marrow aspirations under general anesthesia from the posterior iliac crest. Fifteen hundred to 2500 ml of bone marrow cells were harvested and suspended in Hank's balanced salt solution with preservative free heparin. After collection, the nucleated cells were harvested from the buffy coat and prepared for either fractionation over the albumin gradient or for immediate storage according to the method described previously [8]. Of the 24 evaluable patients in 10 cases unfractionated bone marrow was stored, and in 9 patients separation of leukemic from normal cells by the albumin gradient was attempted.

III. Fractionation of Bone Marrow Cell Suspensions by Discontinuous Albumin Gradients

Erythrocyte poor bone marrow cells were suspended in 17% albumin solution and pipetted on top of more dense albumin (BSA: bovine serum albumin, fraction 5, Sigma) layers consisting of 21%, 23% and 25% albumin solutions of defined osmolarity. These fractions were then centrifuged for 30 minutes at 10° C in an International centrifuge at 2,000 rpm corresponding to 1,000 g at the bottom of the tube. After centrifugation, distinct layers of cells were visible in the gradient near the interfaces between the different albumin layers. The cell fractions between the 17 and 21% BSA layers have been labeled fraction 1+2 between 21 and 23% BSA fraction 3 and so on to fraction 5 at the bottom of the tube. Each fraction was then collected and diluted with Hank's balanced salt solution. Detailed information of the fractionation procedure has been published elsewhere [4].

IV. Bone Marrow Storage and Thawing

Hemopoietic cells were stored according to the technique described by Schaefer et al. [1]. In short, bone marrow cells were suspended in Hank's balanced salt solution (305 mm). After addition of 10% DMSO and 20% calf serum to the cell suspension, the cells were transferred to 2 or 5cc polypropylene ampoules and cooled at 1° C per minute to −40° C, using a cryoson automatic controlled freezer. After rapid cooling from −40° C to −80° C, the cells were stored in liquid nitrogen at −192° C. The cell concentrations during freezing were kept between 20 to 200×10^6 cells per ml. The cell viability was tested immediately after storage and compared with the unfrozen cells using the in vitro colony forming assay for myeloid progenitor cells (CFU-C assay) (see below).

At the time of transplantation the ampoules were thawed rapidly in a 50° C waterbath. Immediately after thawing the cells were slowly diluted with Hank's balanced salt solution. Quantities of Hank's balanced salt solution equivalent to 1/50, 1/25, 1/12, etc. of the ampoule volume were added dropwise until the original volume was diluted 10-fold. Between each dilution step, the suspension was carefully mixed for 3 to 5 minutes. Routinely, DNase (Deoxyribonuclease, Sigma 4 mgr, 7600 U, per 500cc collected marrow) was added to avoid clump formation by extracellular DNA [21]. After stepwise dilution, the cells were centrifuged, resuspended in Hank's and filtered through G_2 glass filters (Jena glass, pore size 40–80μ). Viability of the cells was again tested by the CFU-C assay.

V. In Vitro Assay for Hemopoietic Stem Cells (CFU-C assay)

Two hundred thousand cells were suspended in 1 ml of agar medium (0.3% agar, α-MEM and 15% fetal calf serum) and pipetted onto previously prepared human peripheral blood leukocyte (1×10^6 ml) underlayers in agar medium (0.5% agar, α-MEM and 15% fetal calf serum). After gelling, cultures were incubated for 7 days and colonies larger than 40 cells were evaluated visually using an inverted or dissecting microscope [20].

VI. In Vitro Assay for Leukemic Cells (PHA assay)

Basically the technique consists of 2 phases; initial liquid phase of 15 hours at 37° C and a semi solid phase of 7 days incubation at 37° C [6, 23]. In the liquid phase 2×10^6 cells/ml medium (α+15% calf serum were cultured in falcon plastic tissue culture tubes) to which 2–4µg PHA (Welcome) per ml was added. After 15 hours of incubation the cells were washed twice using Hank's balanced salt solution (305 mOsm) and resuspended in agar medium (final concentration in agar 0.25% medium + 15% calf serum). After resuspending in agar medium, the cells 1×10^5 per ml per dish were pipetted in falcon plastic petri dishes containing 1 ml agar medium to which PCM ranging from 0.1 to 0.25 ml per culture dish was added simultaneously. The cells were plated in petri dishes containing agar underlayers without PCM. After 7 days of incubation in 7.5% CO_2 gas controlled humidified incubators at 37° C, colonies were visible microscopically. These colonies were counted using an inverted or dissecting microscope. Aggregates containing 40–50 cells or more were considered colonies, aggregates containing less than 40–50 cells are considered to be clusters.

VII. Conditioning of the Patient for Bone Marrow Transplantation

Prior to bone marrow transplantation, the patients were treated with i.v. administration of piperazinedione (NSC 135785), 25 mg/m^2 administered on days 6 and 5 before TBI. In the first 17

patients 850–950 rad calculated at the mid abdominal plane were delivered at a dose rate of approximately 12–14 rad per minute using a 25 MEV linear accelerator. In the following 6 patients, 750 rad were administered and in the last 5 patients the TBI was administered in 6 fractions of 200 rad (200 rad twice daily over 3 days: total dose 1200 rad). The patients were treated horizontally with ¼ of the total dose being delivered from the anteriour, posterior, right and left lateral aspects to minimize dose inhomogeneity. Within 24 hours after TBI nucleated cells of fractions 3, 4 and 5 were infused.

D. Results

I. Results of Separation of Normal Hemopoietic Cells from Leukemic Cells

Separation between leukemic cells and normal cells using the discontinuous gradient technique was demonstrated in three different experimental settings. In the first experimental setting, bone marrow cells from patients were separated which consisted predominantly of normal hemopoietic cells and a small but detectable percentage of leukemic cells. As monitor assay for normal hemopoietic cells, the CFU-C assay was used and for the leukemic cell population cytogenetics and electromicroscopy. In Table 1 the results have been documented. Patient DH had an aneuploid leukemic cell clone and therefore, cytogenetic analysis was possible. It can be seen in Table 1 that in fractions 1+2 and 3, 3 respectively 4 of the 20 analysed metaphases revealed the abnormal karyotype whereas in fractions 4 and 5 this karyotype could not be found. Electronmicroscopical (EM) analysis was positive for leukemic cells in fractions 1+2 and 3 and negative in 4 and 5. The nuclear pocket in the cell is characteristic for leukemic cells with abnormal karyotypes [1]. The CFU-C population was predominantly in fractions 4 and 5: 64% so that the majority of this population was deprived of detectable numbers of leukemic cells suggesting separation between the two cell populations. The second experimental setting in which evidence could be provided of separation between normal cells and leukemic cells, was analysis of so called "remission marrow" (with morphologically undetectable number of leukemic cells) using the PHA assay as parameter for leukemic cells. In Table 2 the results were depicted. Analysis of the fractions revealed positive PHA assay in fraction 1+2 whereas in the other fractions, cells which were able to give rise to colonies after PHA assay in fraction 1+2 whereas in the other fractions, cells which were able to give rise to colonies after PHA stimulation, could not be demonstrated. Morphological analysis of the colonies revealed the presence of

Table 1. Separation between normal stem cells and leukemic cells in remission marrow

	CFU-C as percentage of total	Leukemic Cell Population	
		Cytogenetics	EM
Fr 1+2	1%	4/20	+
Fr 3	35%	10/20	+
Fr 4	48%	0/20	−
Fr 5	16%	0/20	−
Unfractionated		2/20	+

Table 2. Distribution of leukemic cell colonies using the PHA assay and of colonies from normal progenitor cells using the Robinson assay in the gradient of marrow cells of a patient in remission

	CFU-C in robinson assay		PHA assay	
	Per 10^5 Plated	% Recovery	Leukemic Cell Colonies /10^5 Cells[b]	Cytogenetic Analysis[c]
Unfractionated	20	100	0	−
Fraction 1+2	20	2	10	+
Fraction 3	58	35	0	+
Fraction 4	40	50	0	−
Fraction 5	5	13	0	−

[a] As percentage of total number of CFU-C put on gradient
[b] Number of colonies in dish containing no leukocyte feeder layer or placenta conditioned medium
[c] 20 metaphases analyse: +abnormal kariotype present, − abnormal karyotype absent

blast cells, which were morphologically identical to the leukemic cell population before chemotherapy treatment. In fraction 1+2 only a small percentage, 2% of the normal CFU-C population was present; the majority of CFU-C were found in fractions 3, 4 and 5. The third experimental setting of separation of leukemic cells and normal cells was obtained by comparing in one patient the leukemic cell profiles in the gradient of the marrow at the time of relapse and the CFU-C profile of the marrow after achieving remission. In Figure 1, those profiles were depicted and it can be noted that the peak activity of the two populations were in different fractions.

II. Results of Marrow Transplantation

Characteristics of the bone marrow grafts have been listed in Table 3. There is a difference in number of cells infused per kg body weight (b.w.) between the fractionated and unfractionated group (median 0.96×10^8 versus median 3×10^8), however, there is no significant difference between the total number of CFU-C infused. The number of cells recovered after freezing ranged from 50 to 75%; the number of CFU-C per 10^5 plated cells is not statistically different before and after freezing, which confirms the previously published data [4]. The two patients who did not show engraftment presented with absence of colony growth at the time of thawing. In those two patients also before freezing the CFU-C number per 10^5 plated cells was low (less than 0.5), which is at least a factor 20 less than the CFU-C concentration in normal bone marrow. The median time interval between storage and subsequent relapse was 9 months, (range 0.5–16) which may reflect the chance of leukemic cell contamination in the stored marrow.

In Table 4, the results of the 28 patients have been listed. All 24 evaluable patients showed clearing of bone marrow from leukemic cells. Twenty-one out of

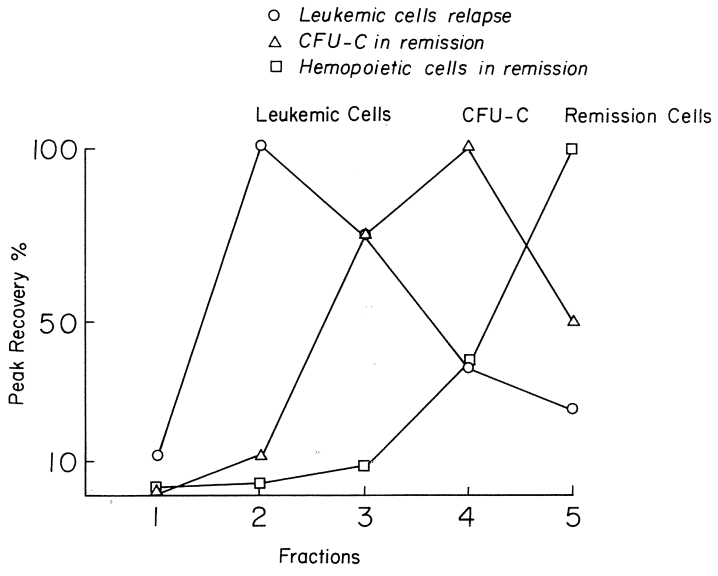


Fig. 1. Density profiles of leukemic cells before treatment and of the CFU-C and bone marrow cells after remission induction. On the vertical axis the peak activity has been depicted, on the horizontal axis the number of the fractions. Note a clear cut difference in the gradient of the peak activity of the leukemic cell population (in fraction 1+2) and of the CFU-C population (in fraction 4). ○ Leukemic cells in relapse; △ CFU-C in remission; □ Hemopoietic cells in remission

24 had evidence of marrow engraftment by the presence of nucleated cells in the peripheral blood. Twelve patients achieved CR, as defined as absence of leukemia for more than one month and hemopoietic recovery with granulocytes $>1{,}000/mm^3$ and platelets $>50{,}000/mm^3$ in the peripheral blood. The pattern of hemopoietic recovery of 12 patients achieving CR is shown in Table 5. Ten patients achieved granulocyte counts of $1{,}500/mm^3$ and platelet counts of

Table 3. Bone marrow graft characteristics (n=21)

	Median	Range
Storage time	12[a]	5–30[a]
Interval between onset of remission and storage	12[a]	2–15[a]
Interval between storage and subsequent relapse	9[a]	0.5–16[a]
Number of unfractionated cells grafted	3×10^{8} [b]	$2.2–5\times10^{8}$ [b]
Number of fractionation cells grafted	0.96×10^{8} [b]	$0.068–2.3\times10^{8}$ [b]
Number of CFU-C grafted per 10^5 cells	5	0–50

[a] In months
[b] Per kilogram body weight

Table 4. Current status of the leukemia transplantation program

Number of patients grafted:	28
Number of evaluable patients:	24
Number of takes:	21
Number of CR:	12
Number of CR patients alive:	3
Median remission duration:	4+months (range: 2–14)
Median survival of CR patients:	6+months (range: 2–14)
Number of relapse patients alive:	2
Number of recovering patients:	1

100,000/mm^3 peripheral blood. In one of the 12 patients (WD), platelet and granulocyte recovery was protracted: platelets counts of >100,000/mm^3 were reached on day 60 and granulocyte counts of >1,000/mm^3 on day 42. This patient had received a low number of cells (6.8×10^6 cells/kg b.w.) of fraction 3, which had a six fold enrichment of CFU-C. In patient (TG) the granulocytes and platelets increased to 1,200/mm^3 and 60,000/mm^3 respectively prior to relapse at day 60. Patient JW reached granulocyte levels of >500 by day 35, >1,000 by day 80 and platelet counts of >20,000 by day 80, >50,000 by day 114 without recurrence of leukemia 5+ months after transplantation. Of the 12 patients who reached CR, 3 are still alive in unmaintained remission: 4+, 4+, 7+ months. One patient relapsed after 2 months and is alive 4 months after relapse with a slow growing leukemic infiltrate, not requiring chemotherapy. Seven patients died: 5 due to recurrence of leukemia and 2 due to infections.

Of the 24 evaluable patients listed in Table 4, 12 did not achieve CR. In 9 out of 12 patients signs of take were evident, but 8 patients died before achieving CR. The causes of death were: adult respiratory distress syndrome (3), cardiomyopathy with intractible congestive heart failure (2), infections (2), and recurrent leukemia (3). One patient (MT) is in the recovery phase after transplantation and

Table 5. Hematopoietic recovery of the eleven patients achieving complete remission[a]

Platelets/mm^3[b]	$\bar{m}$	Range
> 20,000	22	(20–80)
> 50,000	39	(24–114)
>100,000	55	(30–65)
Granulocytes/mm^3		
> 500	21	(15–35)
>1,000	28	(17–80)
>1,500	42	(21–60)

[a] 2 patients (TG, JW) did not achieve a granulocyte level of >1,500/m^3 and platelets >100,000/mm^3 in the peripheral blood

[b] In the peripheral blood

	Unfractionated group	Fractionated group
CR:	7/12	5/10
Duration:	2+, 2, 2+, 4, 6+, 7, 9 months	2, 2, 3, 6+, 14 months

Table 6. Comparison of CR and duration of CR of patients transplanted with unfractionated or fractionated marrow

at the time of writing the granulocyte count is $>1{,}000/mm^3$ and the platelets are $45{,}000/mm^3$. There is no evidence of leukemia.

As can be seen in Table 6, of the 24 evaluable patients, 10 patients were transplanted with fractionated marrow cells. Of these 10 patients, 5 achieved CR with durations of 2, 2, 3, 6+ and 14 months. These data are not statistically different from the results of the 14 patients transplanted with unfractionated bone marrow: 7 achieved CR with a duration of 2, 2+, 3, 4, 6+, 7, 9 months. The hemopoietic recovery in the two groups was not different.

	N	C.R. Rate
First Remission		
ALL/AUL	5	4
AML	13	8
Second Remission		
ALL/AUL	4	0
AML	2[a]	0

[a] One patient in recovery phase

Table 7. Comparison of remission rate after BMTR between patients with marrow collected in first remission and in second remission

Table 7 shows the influence of timing of bone marrow aspiration on the outcome of the bone marrow transplantation. All 12 patients who achieved CR had their bone marrow aspiration during the first remission. Of the 6 patients who had their bone marrow aspirated during second remission, as yet none achieved CR although in 4 patients signs of engraftment were noted and another patient is in the recovery phase after transplantation. It can also be seen that Table 8 demonstrates that when bone marrow transplantation was given as the first treatment after occurrence of a relapse, 7 out of 12 patients achieved CR (68%), which is slightly higher than a 41% remission rate (5 out of 12) when second line chemotherapy was given for treatment of AML in first relapse and of ALL in second relapse before entering the bone marrow transplantation program.

		N	C.R.	
BMTR As First Line Therapy In Relapse	AML	12	7	68%
	ALL	–	–	
BMTR As Second Line Therapy In Relapse	AML	3	1	41%
	ALL	9	4	

Table 8. Influence of pretreatment of relapse on CR rate after BMTR

E. Discussion

Case reports of relapsed acute leukemia treated with high dose cytoreductive regimens and autologous remission bone marrows have been published by several authors and document that this is at least a method in which CR can be achieved [14, 15, 17, 22]. Our study evaluated autologous transplantation following high dose chemotherapy and supralethal TBI as an integral part of anti-leukemia therapy for AML in first relapse and ALL and AUL in second or third relapse. At the time of analysis, 201 remission bone marrows have been stored over a time period of three years.

Autologous transplantation in relapsed acute leukemia involves specific problems. The questions we were asking at the start of our program concerned the survival of pluripotent stem cells over prolonged periods of cryopreservation, the transplantation potential of bone marrow stem cells exposed to continuous chemotherapy, the persistence of leukemic cells in the remission bone marrow and their possible removal by albumin density gradient centrifugation, and the development of an optimal cytoreductive regimen with limited nonhematopoietic toxicity.

Bone marrow stored for up to 30 months in liquid nitrogen maintained its transplantation potential and allowed for full hemopoietic recovery after piperazinedione and TBI. The pattern of hemopoietic recovery with granulocyte levels above 500 and platelet levels above 20,000 by 3 weeks appeared similar to that reported in the syngeneic bone marrow transplantation studies by the Seattle group [9].

Failure of bone marrow take correlated with absence of colonies in the CFU-C assays in 2 cases. One patient died at day 30 due to fungemia and intractable congestive heart failure with no evidence of a take by bone marrow examination and peripheral blood count, the second patient died at day 56 after transplantation due to disseminated CMV infection without evidence of hemopoietic recovery. This experience suggests that the CFU-C assay might be a valuable monitor or the quality and viability of cryopreserved bone marrow. Recent analysis of our culture data showed that a significant correlation exists between the number of CFU-C infused and the time of recovery of granulocytes [24].

In the separation studies it is evident that the leukemic cell population is predominantly lighter than the normal CFU-C population and that the leukemic cell population can be reduced by a factor of up to 10 in the cell suspension to be grafted. These results confirmed the data of Moore et al. [18]. In 10 patients fractionated cells were used for transplantation. At the time of writing no difference exists in rate as well as duration of CR between the patient group treated with fractionated marrow and the group infused with unfractionated marrow. Although the numbers are small and therefore definite conclusions can not be drawn, the results may indicate that a 1-log reduction of infused leukemic cells has no biological significance. Further improvement of density separation and addition of other separation techniques hopefully reduce the number of leukemic cells in the graft even more. It remains to be seen if even complete or nearly complete elimination of leukemic cells from the graft will reduce the

incidence of occurrence of leukemia since recurrence of leukemia after transplantation may well be caused predominantly by the leukemic cell population escaping the chemo-irradiation therapy. There has been no significant difference noted in terms of hematopoietic recovery in the patients who received unfractionated bone marrow and in those who received gradient separated bone marrow fractions. It is possible to assess the degree of separation between leukemic ells and normal remission cells in any individual patient. Since there are differences in the densities of leukemic cells it seems appropriate to study the leukemic cells at the time of first presentation and compare the pattern of distribution with that of CFU-C in remission to see if separation is possible. In one patient (KL) leukemia rapidly recurred after initial clearing of the bone marrow. Leukemia became evident 30 days after transplantation. In this case the marrow has been aspirated in the second remission, 2 weeks prior to overt relapse. The unfractionated marrow was transplanted after all other means of therapy were exhausted. It is conceivable that in this case leukemia was transplanted [5].

Total body irradiation or chemotherapy alone used as cytoreductive regimen in bone marrow transplantation studies has led to limited remission durations [26]. The combination of chemotherapy and irradiation yielded a higher percentage of long term surviving patients [26]. The actuarial relapse rate of patients treated with the SCARI regimen, a highly toxic chemo-irradiation treatment consisting of 6-Thioguanin, Cytoxan, Ara-C, Daunorubicin and irradiation is lower than the actuarial relapse rate with Cytoxan/TBI [12]. However, the percentage of patients surviving more than two years is not significantly different [12]. For our conditioning regimen we selected a combination of piperazinedione, a fermentation product of a streptomyces species with alkylating activity [13] and TBI. In all evaluable cases, the size of the leukemic cell population was reduced to morphologically undetectable levels, but follow-up after transplantation is too short to draw definite conclusions about effeciency of leukemia eradication compared to the other regimens used. Clinical studies have shown that piperazinedione had good anti-leukemic activity but its general use was abandoned because of excessvie myelotoxicity [3]. Non-hematopoietic toxicity of this drug is minimal and restricted to mild nausea and vomiting. The interval of 5 days between piperazinedione and TBI was necessary as close proximity of piperazinedione and TBI led to excessive gastrointestinal toxicity in the mouse [7].

The toxicity of this program is in part related to cumulative effect of preceding chemotherapy. The two patients who developed intractable congestive heart failure with cardiomyopathy on autopsy had received large amounts of anthracyclines 500 mg/m^2 Adriamycin in one case and 1,750 mg/m^2 of Rubidazone in another patient. The acute pulmonary toxicity occurred in 3 patients who had received more than average amounts of chemotherapy in the pre-transplantation course and high dose irradiation at the time of transplantation (950, 900 and 850 rad). In an attempt to decrease the acute radiation toxicity we reduced TBI to 750 rad. Four of 6 patients showed CR (MB, SE, JO, JW), 2 patients recurred with leukemia (BP, WW). Less acute toxicity was seen with this regimen. In order to further reduce toxicity we started to administer TBI in fractionated doses of 200 rad twice daily for three days. Theoretically, 1200 rad

fractionated irradiation has similar leukemic cell cytoreductive capacity as 800 rad bolus irradiation [19].

At this time it seems that piperazinedione and TBI followed by autologous bone marrow transplantation is an effective treatment for relapsed acute leukemia and leads in about half of the cases to unmaintained CR. The quality of life of patients achieving CR is good. The anti-leukemic efficacy of this chemo-irradiation program in conjunction with autologous bone marrow transplantation needs to be evaluated further.

It becomes clear from the evaluation of the effect of timing of bone marrow aspiration and of preceding chemotherapy on response to bone marrow transplantation that this procedure is effective when it is employed early in the course of relapse: 11 out of 17 patients who underwent earlier bone marrow transplantation achieved CR. Eleven of the 12 patients who did not achieve CR, reflect cumulative drug toxicity, decreased transplantation potential of the marrow, stored after extensive exposure to chemotherapy and poor clinical condition after failing reinduction with chemotherapy.

At the time of writing, the anti-leukemic effect of piperazinedione and TBI in conjunction with autologous bone marrow transplantation is promising, leading to CR in approximately 50% of the cases. The quality of life after transplantation is good and the hospitalization time after transplantation of the patients achieving hemopoietic recovery is relatively short (median 26 days). More patients treated earlier in relapse are needed to compare this treatment modality in terms of survival with patient groups treated with conventional chemotherapy.

References

1. Ahearn, M. J., Trujillo, J. M., Cork, A., Fowler, A., Hart, J. S.: Cancer Res. *34,* 2887 (1974)
2. Benjamin, R. S., Keating, M. J., McCredie, K. B., Bodey, G. P., Freireich, E. J.: Cancer Res. *37,* 4623 (1977)
3. Benjamin, R. S., Keating, M. J., Valdivieso, M., et al.: Submitted to Cancer Treat. Rep. (1978)
4. Dicke, K. A., et al.: Experimental Hematology Today (In Press)
5. Dicke, K. A., McCredie, K. B., Stevens, E. E., et al.: Transplant. Proc. *9(1),* 193 (1977)
6. Dicke, K. A., Spitzer, G., Ahearn, M. J.: Nature *259(5539),* 129 (1976)
7. Dicke, K. A., Scheffers, H. M., Mason, K. A., et al.: Abstract, Internation Society for Experimental Hematology, Fourth Annual Conference 1975, Yugoslavia, 30, 67, September 21–24, 1975
8. Dicke, K. A., Tridente, G., van Bekkum, D. W.: Transplantation *8,* 422 (1969)
9. Fefer, A., Thomas, E. D., Buckner, C. D., et al.: Semin. Hematol. *11,* 353 (1974)
10. Freireich, E. J., Keating, M. J., Gehan, E. A., et al.: Cancer *42,* 874 (1978)
11. Gale, R. P., Cline, M. J.: Lancet 497, March 5 (1977)
12. Gale, R. P.: The UCLA Bone Marrow Transplantation Team. Transplant. Proc. *10(1)* (1978)
13. Gitterman, C. O., Rickes, E. L., Wolf, D. E., et al.: J. Antibio. *23(6),* 305–310 (1970)
14. Gorin, M. C., Najman, A., Duhamel, G.: Lancet 1050 (1977)
15. Herzig, G. P.: Personal Communication
16. McCredie, K. B., Hewlet, J., Gehan, E.: Abstracts Vol. I, 1978. XVII Congress of the International Society of Hematology, Paris, 231, 1978
17. McGovern, J. J., Russell, P. S., Atkins, L., et al.: N. Engl. J. Med. *260,* 675 (1959)
18. Moore, M. A. S., Williams, N., Metcalf, D.: J. of the NCI *50,* 591 (1973)
19. Peters, L. J., Withers, H. R., Cundiff, J. H., Dicke, K. A.: Accepted for publication, Radiology (1979)

20. Pike, B. L., Robinson, W. A.: J. Cell. Physiol. *76,* 77 (1970)
21. Schaefer, U. W., Dicke, K. A., van Bekkum, D. W.: Rev. Europ. Etud. Clin. et Bio. *17,* 483 (1972)
22. Schaefer, U. W., et al.: Exp. Hematol. *5(2),* 101 (1977)
23. Spitzer, G., Dicke, K. A., Schwartz, M. A., Trujillo, J. M., McCredie, K. B.: Blood Cells *2,* 149 (1976)
24. Spitzer, G., Verma, D. S., Zander, A., et al.: Blood *52(5),* 232 (1978)
25. Thomas, E. D., Buckner, C. D., Banaji, M., et al.: Blood *49(4),* 511 (1977)
26. Thomas, E. D., Storb, R., Clift, R. A., et al.: N. Engl. J. Med. *292,* 832 (1975)
27. ULCA Bone Marrow Transplantation Team: Lancet *1197,* December 10 (1977)

Discussion

Kaizer: In view of the heavy previous treatment of your patients at the M. D. Anderson Hospital your results are surprisingly good. Have you tried your regimen in patients with twin transplants and allogeneic transplants and what were your results?
Dicke: Yes, with allogeneic transplants in these heavily treated patients only 2 of 17 survived to be at risk of leukemia recurrence and these two did not get leukemia.
Haas: Why did not you graft your patients in remission?
Dicke: This is a phase I study, but I think that we ought to combine every treatment modality in the future and graft the patient in remission.
Fliedner: I think that the fractionation studies are principally very importent. It has been discouraging that you found no difference with and without fractionation of the marrow. Have you calculated the amount of leukemic cells remaining in the body and compared it to the few cells in the graft? This is an important consideration.
Dicke: Yes, this is important and my plans are: first give unfractionated marrow and the second time take marrow in remission and fractionate it.
Thiel: Did you test your gradient in an animal leukemia model?
Dicke: Yes, Dr. Hagenbek tested it in the BN-rat leukemia model and we found a 2–3 log reduction, but we can not conclude from this on the human situation.
Prentice: What rate of patients suffered from respiratory distress syndrome?
Dicke: We have seen this in patients who have got 900–950 rad in about one hour and that is the reason we decreased the dose to 750 rad.

7 Conditioning of Bone Marrow Recipients

Mechanisms of Transplantation Tolerance to Allogeneic Bone Marrow Cells Following Total Lymphoid Irradiation (TLI)

S. Slavin and S. Strober

A. Introduction

I. The Principles of Total Lymphoid Irradiation (TLI)

The lymphocytes, the mediators of cellular events that lead to subsequent allograft rejection, are very radiosensitive, being particularly vulnerable to irradiation during interphase. Nevertheless, sublethal doses of whole body irradiation (WBI) cannot be used as the sole agent for immunosuppression for prevention of allograft rejection due to quick recovery of lymphocyte number and function following WBI. The WBI dose required to ensure engraftment of bone marrow (BM) or organ allografts is intolerable due to irreversible detrimental effects on radiosensitive organs like the BM, the gastrointestinal tract and the lungs. Fractionated, high dose total lymphoid irradiation (TLI) was originally designed to ensure maximal lymphoid depletion and avoiding unnecessary exposure of nonlymphoid organs, using selective ports of irradiation directed to the lymphoid organs.

The TLI procedure was developed on the basis of long term observation previously reported by Fuks et al. (1976) in patients with Hodgkin's disease following radiotherapy. Total nodal irradiation, a routine radiotherapeutic procedure for the treatment of malignant lymphomas (Kaplan, 1972) resulted in longlasting thymusderived (T) lymphocytopenia as well as impairment of T cell function (Fuks et al., 1976).

TLI involves two major principles:

1. The use of selective ports of irradiation directed to the lymphoid organs including the major lymph nodes, thymus and spleen with lead shielding of nonlymphoid organs. In order to obtain the maximal immunosuppressive effects of TLI it is essential to include the thymus in the irradiation field (Slavin et al., 1977, 1978b; Zan-Bar et al., 1979).
2. Fractionation of the irradiation into daily doses of well tolerable fractions of 100–200 rads/day, achieving high total cumulative radiation doses with relatively minimal side effects. In rodents TLI consists of 17 daily fractions of 200 rads, a total cumulative dose of 3400 rads.

The ports of irradiation must be carefully designed and modified for each species, using conventional simulation techniques. The same lead apparatus can be used only for animals of similar sizes (like standard sized adult mice and rats). Application of TLI to larger animals (dogs, primates) and man requires individual lead shield designs using a simulator. Further details for the TLI procedure were previously reported in mice (Slavin et al., 1976, 1977), rats (Slavin et al., 1978a), and dogs (Slavin et al., 1979).

II. Effects of TLI on the Murine Immune System

TLI (200 rads × 17, total cumulative dose of 3400 rads) has a profound effect on the humoral as well as the cell mediated immune responsiveness of BALB/c (H-2^d) mice. The primary T-dependent response to sheep red blood cells (SRBC) is totally eliminated for one month following termination of TLI. IgM response begins to return by day 44 and the primary IgG response at around 200 days. The secondary response remains considerably lower than the normal secondary anti-SRBC response for more than 9 months following TLI (Zan-Bar et al., 1979).

TLI does not eliminate memory cells, as indicated by good secondary responses to SRBC following TLI, in BALB/c mice that were immunized to SRBC prior to TLI (Zan-Bar et al., 1979).

The effects of TLI on the T-cell mediated immune function can be demonstrated both in vitro and in vivo. T-lymphocyte numbers as well as in vitro T lymphocyte functions including proliferative responsiveness to phytohemagglutinin (PHA), Conconavalin A (Con A) and responsiveness to allogeneic lymphocytes in a one way mixed lymphocyte reaction (MLR) are totally eliminated for one month following TLI. Longterm kinetic studies on the recovery patterns following TLI in BALB/c mice reveal persistent T lymphocytopenia and compensatory B lymphocytosis, complete recovery of MLR reactivity, impaired responsiveness to PHA and enhanced responsiveness to Con A. The in vivo correlate of the impaired cell mediated immune state following TLI is a prolonged survival of a strongly histo-incompatible C57 BL/Ka (H-2^b) skin allograft (mean survival 49.1 days compared to 10.7 days in untreated controls, Slavin et al., 1977).

III. Antigen-Specific Tolerance Induction in Mice Following TLI

Antigen specific tolerance to soluble protein antigens (bovine serum albumin, BSA and bovine gamma globulin, BGG) could be established by injecting the nondeaggregated antigens into TLI treated BALB/c mice. Tolerant BALB/c mice fail to generate anti-DNP antibody response to a challenge of DNP-BSA or DNP-BGG in complete Freund's adjuvant, respectively, six weeks later. Spleen cells from unresponsive mice suppress the adoptive secondary anti-DNP response of sublethally irradiated syngeneic hosts given BSA or BGG primed T cells, DNP-BSA or DNP-BGG primed B cells, and DNP-BSA or DNP-BGG in saline as a secondary challenge, respectively. Suppression is antigen specific and mediated by Thy 1.2 positive cells, suggesting that host type specific suppressor T cells are involved in the mechanism of antigen specific tolerance following TLI (Zan-Bar et al., 1978).

B. Allogeneic BM Transplantation Following Conditioning with TLI

Bone marrow transplantation across major histocompatibility barriers was accomplished in 3 animal models tested so far including inbred mice and rats, and mongrel dogs (Table 1). Engraftment was accomplished following intravenous

Table 1. Evidence for chimerism in various cell compartments in different species transplanted with allogeneic bone marrow following total lymphoid irradiation (>6 months following transplantation)

	Donor Type Cells				
	Peripheral Blood Leukocytes	Bone Marrow	Spleen	Lymph Nodes	Red Blood Cells
Mice, C57BL/Ka, $H2^{b/b}$ → BALB/c, H-$2^{d/d}$	44–91%[a]	29[a,b]–80%[a,c]	88%[a]	95%[a]	+[d]
Rats, ACI, $AgB^{4/4}$ → Lewis, $AgB^{1/1}$	48–97%[a]	ND	ND	ND	ND
Dogs, Mongrel ♂ → Mongrel ♀	+[e]	+[f]	ND	ND	+[g]

[a] Tested using a complement dependent microcytotoxicity assay with specific alloantisera (Slavin et al., 1977, 1978a, 1978b)
[b] Bone marrow derived from bones shielded to irradiation (Slavin et al., 1977)
[c] Bone marrow derived from bones exposed to irradiation (Slavin et al., 1977)
[d] Tested using a microhemagglutination assay with specific alloantisera (Slavin et al., 1977)
[e] Documented by presence of donor type DLA antigens in peripheral blood cells (kindly tested by Dr. R. Storb, Fred Hutchinson Cancer Research Center, Seattle, Washington, D.C.)
[f] Assayed using a karyotype analysis of a colchicine inhibited spontaneous metaphase spread of a bone marrow aspirate (Slavin et al., 1979a; Strober et al., 1979)
[g] Tested using a hemagglutination assay with anti-DEA alloantisera (kindly supplied by Dr. R. W. Bull, Department of Medicine, Michigan State University, Michigan, U.S.A.) (Slavin et al., 1979a; Strober et al., 1979)
ND = not done

administration of sufficient amounts of BM cells one day following TLI. Chimerism could be confirmed by documentation of donor-type red and white cells in different body compartments using different techniques (Table 1). Studies on the state of chimerism in mice following TLI indicate that donor type cells migrate predominantly to spaces that were exposed to irradiation including lymph nodes, spleen and irradiated bones. Chimerism, once established is permanent (>360 days).

The most intriguing observations on the TLI model involves the lack of clinical signs of graft versus host disease (GVHD) in mice, rats and canine chimeras, despite the strong histoincompatibilities between the BM donor and the recipients (Slavin et al., 1978, 1978a, 1978b, 1979; Strober et al., 1979). However, administration of equal amounts of allogeneic spleen cells to mice treated with TLI results in lethal GVHD, indicating that TLI protects the recipients from BM-induced GVHD, the so called "secondary disease", but not from the acute phase of GVHD induced by immunocompetent spleen cells (Slavin et al., 1978b).

C. State of Tolerance to BM-donor-type Alloantigens Following TLI

Evidence for Permanent and Specific Tolerance to BM-Donor-Type Alloantigens in Rodents

1. Permanent and Specific Tolerance to Organ Allografts, in vivo Studies

Recipients of allogeneic BM cells following TLI develop permanent and specific transplantation tolerance to organ allografts derived from animals syngeneic to the BM donors. Thus, BALB/c mice develop permanent (>360 days) tolerance to C57BL/Ka skin allografts following pretreatment with TLI and C57BL/Ka BM infusion, despite a full recovery of their ability to reject third party C_3H/He ($H\text{-}2^k$) skin allografts (Slavin et al., 1977, 1978b). Similarly, Lewis rats (AgB^1) accept perfused ACI (AgB^4) heart allografts and ACI skin allografts permanently (>360 days) following TLI and subsequent infusion of ACI BM cells, although their ability to reject third party Brown Norway (AgB^3) skin allografts is fully recovered (Slavin et al., 1978a).

It is of interest that permanent survival of ACI heart allografts was observed in Lewis recipients that received only $75\text{–}100 \times 10^6$ BM cells per animal using a modified lead apparatus with smaller irradiation fields (as compared to $100\text{–}300 \times 10^6$ BM cells with a larger irradiation field used for skin allograft recipients) although no significant levels of donor type cells were detectable in the blood of the recipients during a one year follow up (Slavin et al., 1978a). The data suggests that administration of donor type BM cells immediately following TLI may be essential for the induction of specific transplantation tolerance. Minimal persisting levels of donor cells may be sufficient, or alternatively, not even required for the maintenance of tolerance state in organ allograft recipients.

2. Specificity of the Tolerance to Alloantigens, in vitro Studies

In vitro tolerance to BM-donor-type alloantigens could be confirmed in chimeric mice and rats using one way MLR systems. Lymphocytes derived from lymph nodes or spleens of 6 month old chimeras failed to respond against normal lymph node lymphocytes of either donor's or recipient's origin. Spleen and lymph node lymphocytes obtained from chimeric mice (C57 BL/Ka→BALB/c) and rats (ACI→Lewis) recovered their ability to respond against unrelated stimulating lymphocytes (C_3H/He, $C_3H.Q$ and BN, respectively, Slavin et al., 1977, 1978a).

D. Mechanisms of Tolerance to Alloantigens Following TLI

The basic mechanism for understanding the state of tolerance in adult chimeric animals and man remains unknown. The assumption that specific transplantation tolerance may be actively maintained by specific suppressor cells is tempting, although not formally proved as of yet. The presence of recirculating suppressor T cells and adoptive transfer of transplantation tolerance to irradiated hosts has been reported previously in neonatally tolerant mice and rats (Kilshaw and Brent, 1977; Rieger and Hilgert, 1977; Dorsch and Roser, 1977; Holan et al., 1978, respectively).

In the subsequent chapter we report our evidence for the generation of nonspecific as well as alloantigen specific suppressor cells following TLI and BM infusion using in vitro and in vivo experimental systems. Generation of antigen-specific as well as non-specific suppressor T cells in BALB/c mice that were made tolerant by a protein challenge following TLI (Zan-Bar et al., 1978) suggests that both types of suppressor cells are of the host origin.

I. Suppression of MLR by Spleen Cells from Allogeneic Chimeras

1. Non-specific Suppressor Cells

BALB/c spleen cells obtained within 36 days following TLI induction of chimerism (using BM from C57 BL/Ka donors) suppressed 89% of the reactivity of related BALB/c versus C57 BL/Ka MLR (Fig. 1). The suppressive activity of spleen cells from the treated mice was documented by co-culturing them with responding and stimulating lymph node lymphocytes and measuring their ability to suppress the proliferative responsiveness of the responding cells as compared to equal number of indifferent (C57BL/Ka×BALB/c)F_1 spleen cells (to compensate for total cell number in the wells), using otherwise, identical experimental conditions (details of the methods used are described in the legend for Figure 1).

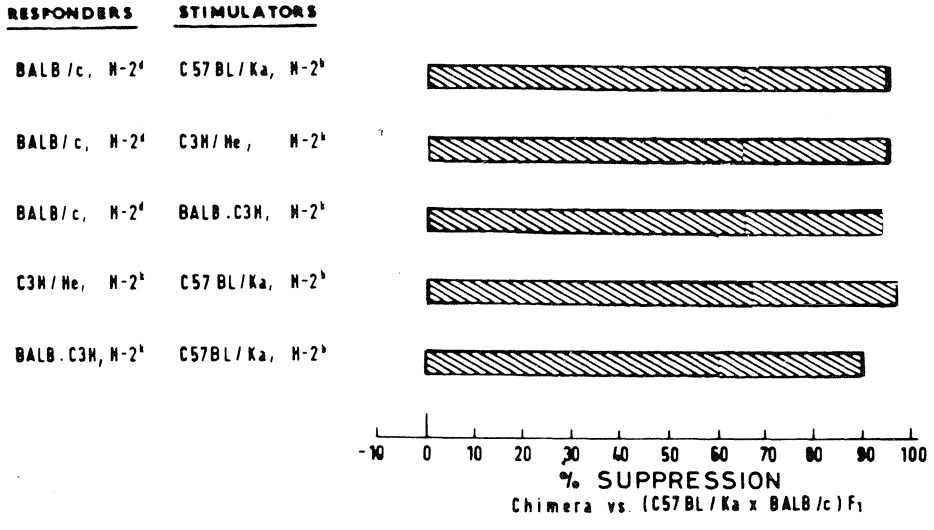


Fig. 1. Studies on the degree of specificity of MLR suppression by spleen cells obtained from BALB/c mice treated with TLI followed by infusion of 30×10^6 C57 BL/Ka BM cells, 30 days following TLI. 10^6 responding lymph node cells and 10^6 stimulating lymph node cells (inactivated by exposure to 3000 rads) were co-cultured with 10^6 spleen cells obtained from chimeras or 10^6 control spleen cells obtained from (C57 BL/Ka×BALB/c)F_1 mice. Co-cultured spleen cells were exposed to 1500 rads prior to culturing to avoid two-way mixed lymphocyte reactivity. The details of the MLR procedure were described previously (Slavin et al 1977). Percent suppression was calculated as (1-^{3}H thymidine uptake of MLR with co-cultured suppressor cells/^{3}H thymidine uptake of MLR with co-cultured (C57 BL/Ka×BALB/c)F_1 cells)×100

The suppressor cells in the spleen were relatively radioresistant since exposure of 1500 rads prior to culture did not eliminate MLR suppression (89% and 95%, respectively). Irradiated spleen cells and (C57 BL/Ka×BALB/c)F_1 controls had to be used for co-culturing with the different one way MLR combinations in order to check the specificity of suppression, thus avoiding 2 way mixed lymphocyte reactivity by the co-cultured cells. The MLR combinatons used are described in Figure 1.

Spleen cells obtained from C57 BL/Ka→BALB/c chimeras within 36 days following TLI and induction of chimerism had the ability to suppress as much as 90–97% of all MLR combinations tested (Fig. 1), indicating that the suppressor cells that were present within the first month following TLI were neither responder nor stimulator-specific (Slavin and Strober, 1979b).

2. Specific Suppressor Cells

Spleen cells obtained from C57 BL/Ka→BALB/c BM chimeras 120–240 days following TLI and induction of chimerism suppressed 81% of BALB/c versus C57 BL/Ka MLR. The suppressive effects of chimeric spleen cells was relatively radioresistant, being 68% following exposure of spleen cells to 3000 rads. MLR suppression by irradiated chimeric spleen cells was specific for both the responding and the stimulating haplotypes as could be demonstrated using different MLR combinations as summarized in Figure 2 (Slavin and Strober, 1979b).

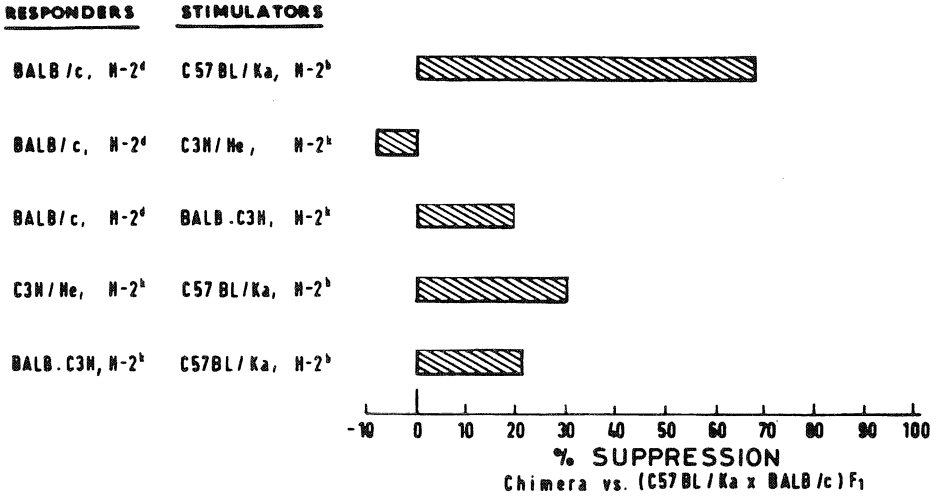


Fig. 2. Studies on the degree and specificity of MLR suppression by spleen cells obtained from C57 BL/Ka→BALB/c BM chimeras 120–240 days following induction of chimerism. The details of the experimental conditions were described in the legend for figure 1. Chimeric spleen cells and (C57 BL/Ka×BALB/c)F_1 controls were exposed to 3000 rads, prior to culturing, to avoid two-way mixed lymphocyte reactivity. Percent suppression was calculated as (1-^{3}H thymidine uptake of MLR with co-cultured suppressor cells/^{3}H thymidine uptake of MLR with co-cultured (C57 BL/Ka×BALB/c)F_1 cells)×100

II. Adoptive Transfer of Specific Transplantation Tolerance and Chimerism by Chimeric Spleen Cells

We have demonstrated that permanent and specific tolerance to skin allografts could be passively transferred across major histocompatibility barriers by adoptive transfer of 25×10^6 spleen cells from C57 BL/Ka→BALB/c chimeras carrying intact C57 BL/Ka skin allografts for more than 6 months into sublethally irradiated (550 rads) syngeneic recipients. Following the adoptive transfer of allogeneic chimeric spleen cells ($\sim$90% H-2^b as typed by specific alloantisera as described previously by Slavin et al., 1977), 7 out of 8 recipient BALB/c mice maintained a C57 BL/Ka skin allograft permanently (>250 days) although they have rejected an unrelated C3H/He skin allograft transplanted concomitantly (mean survival 16.1 days). The adoptive transfer of tolerance to skin allograft was dose dependent since only 2 out of 5 BALB/c mice receiving 12.5×10^6 chimeric spleen cells maintained permanent tolerance to C57 BL/Ka skin allografts. A second passage of 25×10^6 spleen cells from adoptive recipients into sublethally irradiated BALB/c recipients failed to establish permanent tolerance to allogeneic skin grafts (Slavin and Strober, 1979b).

The spleen inoculum adoptively transferred into sublethally irradiated BALB/c recipients failed to induce any clinical signs of GVHD although we have proved that it contained mostly donor type (H-2^b) cells. All sublethally irradiated recipients of similar (25×10^6) or even half (12.5×10^6) this number of allogeneic spleen cells died as a result of GVHD within 33 days (mean survival 11.0 days following administration of 25×10^6 C57 BL/Ka spleen cells; 20.5 days following administration of 12.5×10^6 C57BL/Ka spleen cels; 8.1 days following administration of 12.5×10^6 C57BL/Ka spleen cells mixed with 12.5×10^6 BALB/c spleen cells). The results indicate that permanent and specific tolerance can be transferred by spleen cells derived from tolerant chimeric mice, and that the chimeric cells produce a new chimera in the adoptive recipient without GVHD depsite the strong incompatibility across the major histocompatibility barriers (Slavin and Strober, 1979c).

E. Conclusions

Based on the above studies we conclude that TLI provides a useful approach for induction of immunosuppression, immunomanipulation, and establishment of permanent and specific tolerance to BM and organ allografts across major histocompatibility barriers. Specific as well as nonspecific suppressor mechanisms are involved in the specific tolerance state as well as in the nonspecific immunosuppressive state following TLI, respectively. Specific suppressor cells may be involved in preventing host verus graft as well as GVHD following TLI.

Acknowledgements

This research was supported by the National Institutes of Health grants Al 15387-01, Al 11313, and United States – Israel Binational Science Fundation grant 1786.

References

1. Dorsch, S., Roser, B.: Recirculating, suppressor T cells in transplantation tolerance. J. Exp. Med. *145,* 1149 (1977)
2. Fuks, Z., Strober, S., Bobrove, A. M., Sasazuki, T., McMichael, A., Kaplan, H. S.: Long-term effects of radiation of T and B lymphocytes in the peripheral blood of patients with Hodgkin's disease. J. Clin. Invest. *58,* 803–814 (1976)
3. Holan, V., Hasek, M., Chutna, J.: Radiosensitivity of suppressor cells in neonatally tolerant rats. Transplantation *25,* 27–30 (1978)
4. Kaplan, H. S.: Hodgkin's Disease, pp. 216–279. Cambridge, Mass.: Harvard University Press 1972
5. Kilshaw, P. J., Brent, L.: Further studies on suppressor T cells in mice unresponsive to H-2 incompatible skin grafts. Transplant. Proc. *9,* 717 (1977)
6. Rieger, M., Hilgert, I.: The involvement of suppressor mechanism in neonatally induced allograft tolerance in mice. J. Immunogenet. *4,* 61 (1977)
7. Slavin, S. S., Strober, S., Fuks, Z., Kaplan, H. S.: Long-term survival of skin allografts in mice treated with fractionated total lymphoid irradiation. Science (Wash. D.C.). *193,* 1252–1254 (1976)
8. Slavin, S., Strober, S., Fuks, Z., Kaplan, H. S.: Induction of specific tissue transplantation tolerance using fractionated total lymphoid irradiation in adult mice: Long-term survival of allogeneic bone marrow and skin grafts. J. Exp. Med. *146,* 34–48 (1977)
9. Slavin, S., Reitz, A., Bieber, C., Kaplan, H. S., Strober, S.: Transplantation tolerance in adult rats using total lymphoid irradiation: Permanent survival of skin, heart, and marrow allografts. J. Exp. Med. *147,* 700–707 (1978a)
10. Slavin, S., Fuks, Z., Kaplan, H. S., Strober, S.: Transplantation of allogeneic bone marrow without graft-versus-host disease using total lymphoid irradiation. J. Exp. Med. *147,* 963–972 (1978b)
11. Slavin, S., Gottlieb, M., Strober, S., Bieber, C., Hoppe, R., Kaplan, H. S., Grumet, C.: Transplantation of bone marrow in outbred dogs without graft vs. host disease using total lymphoid irradiation (TLI). Transplantation *27,* 139–142 (1979a)
12. Slavin, S., Strober, S.: Induction of allograft tolerance after total lymphoid irradiation (TLI): Development of suppressor cells of the mixed leukocyte reaction (MLR). I. Immunol. *123,* 942–946 (1979b)
13. Slavin, S., Strober, S.: Transfer of transplantation tolerance by spleen cells from chimeric mice prepared with total lymphoid irradiation. Submitted for publication (1979c)
14. Strober, S., Slavin, S., Fuks, Z., Kaplan, H. S., Gottlieb, M., Bieber, C., Hoppe, R. T., Grumet, F. C.: Transplantation tolerance after total lymphoid irradiation. Transplant. Proc. *11,* 1032–1038 (1979)
15. Zan-Bar, Slavin, S., Strober, S.: Induction and mechanism of tolerance to bovine serum albumin after total lymphoid irradiation. J. Immunol. *121,* 1400–1404 (1978)
16. Zan-Bar, I., Slavin, S., Strober, S.: Effects of total lymphoid irradiation (TLI) on the primary and secondary antibody response to sheep red blood cells. Cell. Immunol. (In press)

Discussion

Tutschka: Your findings of suppressor cells are similar to ours in rats given total body irradiation and allogeneic marrow as you also showed first nonspecific and later specific suppression. Do you know whether your suppressor cells are host of donor? How fast does immunocompetence recur? Is the immunocompetence maintained by nonspecific suppression? In our hands, suppression "in vivo" is relatively radio resistant, whereas "in vivo" suppression can be ablated with 400–500 rads and GVHD reoccurs.

Slavin: In the marrow grafted animals we don't know whether suppressor cells are of donor or host origin.

We know that they are of host origin in the tolerance against BSA and we assume that they are also of host origin in the bone marrow transplantation system. The ability to reject third party skin allografts and MLC reactivity is totally recovered after 70 days, the response to PHA and Con A is normal after 1 month.

Kolb: The limiting toxicity of radiation in whole body irradiation is gastrointestinal toxicity. Did you include the abdomen in the radiation field? What were the total doses of radiation? What was the extent of the chimerism? Was it a split chimerism? Did you compare chimerism in irradiated and unirradiated areas? In mice, did you transfer cells of chimeras back to the original donor strain?

Slavin: In dogs, monkeys and man we irradiated in inverted Y, but in rodents we included the abdomen. In the first experiments with dogs, we gave up to 5000 rads in the mantle field, in the lower part we gave 3000–4000 rads. In the second group of dogs we radiated the whole field in continuity. In this group we gave 1800 rad and in Minnesota 2700 rad without gastrointestinal toxicity. In dogs the chimerism was mixed with mixture of donor red cells in the blood and about 20 per cent donor karyotypes in the bone marrow. In rodents chimerism depended on the cell dose, in lymphoid organs nearly all cells were of donor origin. In irradiated bones like humerus most cells were of donor origin, in the shielded areas like femor most were of host origin. Finally, we have not yet done transfer experiments to the donor strain.

Thierfelder: You showed that spleen cells instead of bone marrow produced GVHD in mice pretreated by total lymphoid irradiation. I would have expected that bone marrow in similarly treated dogs and monkeys would also produce GVHD.

Slavin: We have not observed GVHD in our dogs nor has the Minnesota group. This may be a quantitative phenomenon since in mice we gave large numbers of spleen cells.

Ivanyi: Do you have information on antibody responses in your chimeras?

Slavin: They respond to sheep red blood cells, but we don't have the whole kinetics.

Riethmüller: Have you tested your suppressor cells with respect to Ly marker, particularly kinetics?

Slavin: In vivo we have not, but we have the information in the BSA system that they are θ positive.

Lohrmann: You found tolerance against BSA following total lymphoid irradiation, but not following total body irradiation. How can you explain this?

Slavin: I cannot explain this, the tolerance may be the result of a delicate balance between suppressor and helper mechanisms. All we know is that the thymus has to be included in the radiation field.

Combined Immunosuppression Using Cyclophosphamide Plus Total Lymphoid Irradiation in Preparation for Allogeneic Marrow Transplantation in Humans*

J. H. Kersey, T. Kim, S. Levitt, W. Krivit, M. E. Nesbit, P. Coccia, Phyllis Warkentin, Maura O'Leary, and Norma K. C. Ramsay

Cellular engineering using allogeneic bone marrow offers an attractive form of therapy for a variety of human immunologic and hematologic disorders. The full potential of allogeneic marrow transplantation is yet to be realized, in large part due to problems associated with graft rejection and graft versus host disease [1–5]. Graft rejection (GR) continues to provide formidable obstacles to allogeneic marrow grafting when cyclophosphamide (CY) alone or CY in combination with other agents is used for pretransplant immunosuppression [1–5]. Graft versus host disease (GVHD) has been reported to occur following all immunosuppression combinations including CY and total body irradiation, reported to date [1–5]. In the present studies, we have attempted to reduce the incidence of GR and GVHD in man using new combinations of CY irradiation, or more recently, CY combined with total lymphoid irradiation. Results to date are the subject of this report.

A. Patients

Patients with aplastic anemia were the subject of this study. Only patients with severe aplastic anemia (using the criteria of the International Aplastic Anemia Study Group) were included. A total of 23 patients were studied. They ranged in age from 21 months to 18 years. Clinical details of most of these patients have been previously reported [5]. Two patients had nconi's anemia, one had posthepatic aplastic anemia, and the remaining 20 had idiopathic aplasia. All 23 patients had been multiply transfused prior to transplantation but none with family member donors.

Twenty-one of 23 patients were transplanted with marrow from major histocompatibility complex (MHC) identical sibling donors. Five of these 21 individuals were "sensitized" based on positive results in the complement mediated cytotoxicity assay using recipients' serum and donor cells or using a relative response index of >2 in the mixed lymphocyte culture. Patients received pretransplant immunosuppression in varying combinations as shown in Table 1.

B. Results

Patients treated prior to 1977 received cyclophosphamide (CY) alone or CY in combination with other agents, e.g., 6-mercaptopurine, antithymocyte globulin, or procarbazine. As shown in Table 2, in patients that received this combination of CY with other chemotherapy followed by MHC matched sibling marrow the

* Supported in part by the following grant Nos. CA-21737, CA-15548, CA-18083, and a contract: No. CB-84261-31, all from the National Institutes of Health

1. *Cyclophosphamide* (CY), 50 mg/Kg/day × 4, either alone or in combination with:
   6-Mercaptopurine (6MP), 500 mg/M^2/day × 5
   Antithymocyte globulin (ATG), 15 mg/Kg/day × 3
   Procarbazine (PZ), 15 mg/Kg/day × 3
2. *Total Body Irradiation* (TBI), 750 rads at 26 rads/minute, using the linear accelerator, in combination with ATG and PZ
3. *Cyclophosphamide + Total Lymphoid Irradiation* (TLI)
   Day −6 CY, 50 mg/Kg
   −5 CY, 50 mg/Kg
   −4 CY, 50 mg/Kg
   −3 CY, 50 mg/Kg
   −2 Rest
   −1 TLI (750 rads total, 26 rads/minute)
   0 Transplant marrow

Table 1. Summary of three immunosuppressive programs – March 1979

rejection rate was 60% and the GVHD rate was 70%. (Several patients received more than one transplant.) As the GR and GVHD rates were relatively high, this regimen was abandoned in early 1977. As shown in Table 2, total body irradiation (750 rads at 26 rads/minute using the linear accelerator) was subsequently used in two patients but resulted in significant problems with stomatitis and interstitial pneumonitis in this small group of patients.

In 1977, a new combination immunosuppressive regimen was developed using high dose CY combined with single dose total lymphoid irradiation. This regimen is shown in Table 1. Details of the irradiation techniques have been published elsewhere [13] but basically include irradiation of the mantle plus inverted Y fields (similar to the fields used for Hidgkin's disease therapy) plus the spleen. Since late 1977, a total of nine patients have received the CY-TLI combination prior to transplantation of MHC matched sibling marrow. As shown in Table 2, seven of nine (78%) patients are alive from >23 to >477 days with a median of >223 days. The two patients who died were as follows: One had idiopathic aplastic anemia and Candida albicans sepsis prior to transplantation. He died 19 days post-transplantation of sepsis from Candida. The second death

Table 2. Three pretransplant immunosuppressive programs for severe aplastic anemia-MHC matched sibling donors – March 1979

	CY (1974–1977)	TBI (1977)	CY + TLI (1977–present)
Living	4/10 (>879, >906, >1016, >1471 days)	1/2 (>670 days)	7/9 (>23, >93, >213, >223, >329, >458, >477 days)
Rejection	6/10[a]	0/2	0/9[b]
GVHD (Gr II–IV)	7/10[c]	1/2	1/9[d]
Interstitial pneumonitis	1/10	1/2	0/9
Stomatitis and parotitis	0/10	2/2	0/9

[a] vs [b] $p = <0.010$ [c] vs [d] $p = <0.015$

was in a patient with Fanconi's anemia. This patient developed severe toxicity from the combined regimen involving skin, liver, and GI tract. In addition, she developed acute GVHD and died at day 20. As shown in Table 2, rejection was not seen in any of the patients who received the CY-TLI combination, followed by matched marrow. This is in contrast to the CY experience where 6 of 10 rejected. This experience is significantly different ($p<0.01$) from the previous experience (CY alone) using Fisher's exact test. GVHD was seen only in one patient and this was the patient with Fanconi's anemia. Previously, GVHD was observed with relatively high frequency (7/10 patients in the CY group and 1/2 patients in the TBI group). Differences between GVHD incidence (Grade II–IV) using CY alone + CY + TLI were significant ($p<0.015$).

All patients received post-transplant immunosuppression; the first five patients (in the CY group) were treated with methotrexate (Mtx) using the Seattle schedule [1]. The remaining patients in all three groups were randomized to receive either Mtx alone or Mtx + ATG + Prednisone (ATG + Prednisone was administered beginning at day seven postgrafting and continuing for 14 days). Thus, most patients in all three groups were evenly divided between Mtx or Mtx + ATG + Prednisone. The one case of GVHD in the CY + TLI combination received Mtx for postgrafting immunosuppression.

As seen in Table 2, interstitial pneumonitis, parotitis, and severe stomatitis were not seen in patients receiving the CY-TLI combination. Previously, stomatitis and parotitis were observed in patients receiving TBI Interstitial pneumonitis was observed in one patient each that received CY and TBI.

Not shown in Table 2 are two patients that received CY-TLI followed by MHC mismtached (i.e., haploidentical) marrow. One of these patients received haploidentical sibling marrow but rejected the marrow and died at 60 days. The second also received haploidentical sibling marrow and subsequently developed severe chronic GVHD and died at 288 days.

C. Discussion

Marrow transplantation in severe aplastic anemia has been limited in application due to problems associated with GVHD and GR. Results of our present protocol for pregrafting immunosuppression using the CY-TLI combination, while still preliminary, demonstrate progress relative to the problem of GR in that none of the patients with MHC-identical sibling donors rejected marrow grafts. The overall objectives of the CY-TLI combination were several. First, we wanted to deliver the immunosuppression within a relatively short period because of the high early morbidity and mortality rate in patients with severe aplastic anemia. Second, we wanted to continue CY because of its known immunosuppressive qualities. Third, in order to reduce the morbidity associated with total body irradiation while hopefully maintaining the immunosuppressive qualities of irradiation, we chose to irradiate only the lymphoid system using essentially the same field used for treatment of patients with Hodgkin's disease. The intent was, thus, to target the irradiation to the major lymphoid organs including the thymus, spleen, and lymph nodes while sparing the brain, the mouth, and oral pharynx and

the lungs. Also, about the same time, Slavin, et al. had reported data indicating that total lymphoid irradiation given to the mouse might result in lower incidence of GVHD, at least when the irradiation was given in a fractionated form [6].

GVHD has continued to be a problem in marrow transplantation in all major transplantation centers. Also, the results of patients treated at Minnesota using CY alone indicated a high incidence of acute and chronic GVHD. The preliminary results of the CY-TLI combination are thus encouraging in that only one patient to date has developed GVHD and this developed in the patient with Fanconi's anemia. One additional Minnesota patient with Fanconi's anemia was prepared with CY alone and this patient also developed severe toxicity from the immunosuppressive combination followed by severe and fatal acute GVHD. While the reduced incidence of GVHD could, in part, be due to the Mtx-ATG-Prenisone combination given postgrafting, we believe this is not the only factor since most patients in the CY group also were on the same GVHD prophylaxis protocol. Difference in degree of relative mismatch, due to low levels of MLC reactivity, sex differences or degree of sensitization do not account for the differences either. The major mechanism responsible for the reduced incidence of GVHD using the CY-TLI protocol remains unknown. One possibility is that this combination results in the generation of either specific or non-specific suppressor mechanisms. Studies to evaluate this possibility are currently underway.

The preliminary results of the CY-TLI combination, while encouraging in MHC sibling combinations, are less satisfactory in "mismatched" (haploidentical) combinations in that one of two patients rejected the marrow and the other developed severe and subsequently fatal GVHD.

In the laboratory, we are currently evaluating the CY-TLI combination as pretransplant immunosuppression in the mouse. Preliminary analysis indicates that the CY-TLI combination is effective immunosuppression for skin and marrow transplantation and comparable to 17 dose TLI for this purpose (Kersey, et al., in preparation). Single dose TLI alone, 5 dose TLI alone, and CY alone were relatively ineffective for pretransplant immunosuppression when H2 incompatible marrow was administered. In the same combinations the incidence of GVHD was extremely low across the H2 barrier when either the 17 dose TLI or CY-TLI combination was used.

In conclusion, the overall Minnesota experience indicates significant improvement in survival and morbidity in marrow transplantation for aplastic anemia in the past two years. The improved results are coincided with the introduction of a new pretransplant immunosuppressive combination using a combination of CY + total lymphoid irradiation. This new immunosuppressive combination has reduced the incidence of graft rejection and graft versus host disease below that seen in our earlier experience. Current plans are to widen this study to include additional patients to determine whether these preliminary results are experienced in large numbers of patients.

Acknowledgements

The authors gratefully acknowledge the assistance of Ms. Sharon Roell, Transplant Coordinator, the nurses of the Bone Marrow Transplantation Unit headed by Ms. Jeanette Mefford, and the assistance of Ms. Lucia Johnson and Ms. Sharon Fahlstrom with the manuscript.

References

1. Storb, R., Thomas, E. D., Weiden, P. L., Buckner, C. D., Clift, R. A., Fefer, A., Goodell, B. W., Johnson, F. L., Neiman, P. E., Sanders, J. E., Singer, J.: One-hundred-ten patients with aplastic anemia (AA) treated by marrow transplantation in Seattle. Transplant. Proc. *10*, 135–140 (1978)
2. Gluckman, E., Devergie, A., Marty, M., Bussel, A., Rottembourg, J., Dausset, J., Bernard, J.: Allogeneic bone marrow transplantation in aplastic anemia-report of 25 cases. Transplant. Proc. *10*, 141–145 (1978)
3. Gale, R. P., Cahan, M., Fritchen, J. H., Opelz, G., Cline, M. J.: Pretransplant lymphocytotoxins and bone marrow graft rejection. Lancet *I*, 170–172 (1978)
4. Storb, R., Prentice, R. L., Thomas, E. D.: Treatment of plastic anemia by marrow transplantation from HLA identical siblings: Prognostic factors associated with graft versus host disease and survival. J. Clin. Invest. *59*, 625–632 (1977)
5. Kersey, J., Krivit, W., Nesbit, M., Ramsay, N. K. C., Coccia, P. F., Levitt, S. H., Kim, T. H.: Combined cyclophosphamide – total body irradiation compared to other forms of immunosuppression for marrow transplantation. Exp. Hem. Today, in press (1979)
6. Slavin, S., Fuks, Z., Kaplan, H. S., Strober, S.: Transplantation of allogeneic bone marrow without graft-versus-host disease using total lymphoid irradiation. J. Exp. Med. *147*, 963–972 (1978)
7. Johnson, A. H., Rossen, R. D., Butler, W. T.: Detection of alloantibodies using a sensitive antiglobulin microcytotoxicity test: Identification of low levels of preformed antibodies in accelerated allograft rejection. Tissue Antigens *2*, 215–226 (1972)
8. Kim, T. H., Kersey, J. H., Sewchand, W., Nesbit, M. E., Krivit, W., Levitt, S. H.: Total body irradiation with a high dose rate linear accelerator for bone marrow transplantation
9. Kraemer, K. G., Neiman, P. E., Reeves, W. C., Thomas, E. D.: Prophylactic adenine arabinoside following marrow transplantation. Transplant. Proc. *10*, 237–240 (1978)
10. Shulman, H. M., Sale, G. E., Lerner, K. G., Barker, E. A., Weiden, P. L., Sullivan, K., Gallucci, B., Thomas, E. D., Storb, R.: Chronic cutaneous graft-versus-host disease in man. Am. J. Pathol. *91*, 545–570 (1978)
11. Fenyk, J. R., Smith, C. M., Warkentin, P. E., Krivit, W., Goltz, R. W., Neely, J. E., Nesbit, M. E., Ramsay, N. K. C., Coccia, P. F., Kersey, J. H.: Sclerodermatous graft-versus-host disease limited to an area of measles exanthem. Lancet *I*, 472–473 (1978)
12. Kersey, J. H., Meuwissen, H. J., Good, R. A.: Graft versus host reactions following transplantation of allogeneic hematopoietic cells. Hum. Pathol. *2*, 389–402 (1971)
13. Kim, T. H., Kersey, J., Khan, F. M., Sewchand, W., Ramsay, N., Krivit, W., Coccia, P., Nesbit, M., Levitt, S. H.: Single dose total lymphoid irradiation combined with cyclophosphamide as immunosuppression for human marrow transplantation in aplastic anemia. Int. J. Radiat. Oncol., Biol. Phys. in press (1979)

Discussion

van Bekkum: In your patients conditioned with Cyclophosphamide, how many did get GVHD?
Kersey: There have been 10 patients grafted, six with engraftment showed some evidence of GVHD.
Dicke: What was the cause of death in your animals given 900 R total body irradiation and syngeneic marrow?

Kersey: There was a variety of causes, among them pneumonitis and cardiac problems.
Vriesendorp: How do you explain that you cannot reproduce the results of Dr. Slavin?
Kersey: To a large extent we did reproduce his results in terms of absence of GVHD and longterm chimerism. However, some animals rejected the skin graft.
Storb: What is the percentage of mice with long term chimerism and tolerance to donor type skin grafts?
Kersey: I can't give you other data than those I have just presented. Indeed, we have problems with toxicity and infections in our mouse colony, but once they are solved we may end up with the same results as Dr. Slavin.
Kolb: You have seen GVHD in 7 out of 14 patients given Cyclophosphamide alone, but you have not seen GVHD in your patients given total lymphoid irradiation in addition.
How can you explain this?
Kersey: The mechanism may be quite similar to that of fractionated total lymphoid irradiation as described by Dr. Slavin. Similar suppressive mechanisms may be operative in single dose total lymphoid irradiation and studies of the mechanisms are currently on their way.
Gordon-Smith: You have seen severe GVHD in your mismatched transplants. Do you think that you give too much immunosuppression, since it seems that the balance of the cells removed and the cells left behind is important.
Kersey: It is difficult to determine how much is too much. One of the 2 mismatched grafts which we have done was rejected and the other patient died of GVHD.
Prentice: I have traced 11 patients with Fanconi anemia grafted with bone marrow, none of them rejected the graft, 9 developed significant GVHD and 8 died of GVHD. Do you think that they are unable to raise suppressor cells?
Kersey: Indeed, we and the Paris group had difficulties with transplantation in Fanconis anemia. In a small meeting regarding Fanconi anemia it was stressed that these patients do not tolerate chemotherapy well, have more evidence of GVHD and may indeed be unable to generate suppressor cells.
Storb: We have grafted 5 patients with Fanconi anemia and two of these are alive and well. All engrafted, one died with infection and one with GVHD. This result is not different from earlier results with aplastic anemia.
Gluckman: In our series only one of 5 patients survived, all had severe GVHD and four died. None rejected the graft. Despite reduction of the dose of Cyclophosphamide to 100 mg/kg in the last patient he had engraftment and suffered of severe GVHD.
Tutschka: Dis you also check histology for GVHD in your patients and did you include chronic GVHD?
Kersey: All signs of acute and chronic GVHD are included.

Bone Marrow Grafting in Aplastic Anemia After Conditioning with Cyclophosphamide and Total Body Irradiation with Lung Shielding

E. Gluckman, A. Devergie, A. Dutreix, J. Dutreix, M. Boiron and J. Bernard

A. Introduction

Total Body Irradiation (T.B.I.) is used in clinical marrow transplantation to obtain a permanent chimerism. The rationale of its use depends on the indications of bone marrow transplantation (B.M.T.).

In acute leukemia, a strong antitumoral as well as an immunosuppressive effect are needed. The dose has to be high and homogeneous to obtain a uniform tumor killing. A dose of 1000 centigrey (cGy) T.B.I. associated with a Cyclophosphamide (CYT) treatment is widely used. The main cause of failure are graft versus host disease (G.V.H.D.) and related infections, Interstitial Pneumonitis (I.P.) and leukemic relapse. In aplastic anemia, the use of T.B.I. is controversial. It is probably not indicated if the patient has not received any transfusion or is not preimmunized against his donor before grafting. In those cases, conditioning with Cyclophosphamide may be sufficient [12, 14]. In the other cases, T.B.I. may be choosen as an immunosuppressive regimen.

We have treated, in the Hospital Saint-Louis bone marrow transplant unit, 45 consecutive aplastic patients with a bone marrow graft provided by an HLA identical sibling. From 1973 to 1977, 27 patients received, as conditioning regimen, Cyclophosphamide with or without Procarbazine (PCZ) and antithymocyte globuline (A.T.G.), according to the Seattle group protocol [14, 17, 19, 21]. The number of rejections was found to be very high (59%). Only two patients who rejected their graft survived, one after a second graft with another HLA identical sibling, the other after partial autologous reconstitution [7]. The follow-up of anti HLA antibodies was a good predictive test of rejection [8].

Seven patients survived with a complete chimerism with a follow-up ranging from 1 to 4 years.

These results show that the main problem was related to the insufficiency of immunosuppression. We therefore used, since 1977, a conditionning regimen derived, with slight modifications, from the regimen used to condition leukemic patients [20]. Eighteen patients received Cyclophosphamide 60 mg/kg for two days associated with a 800 cGy total body irradiation with a 400 cGy lung shielding. The results of this new conditioning regimen will be reported in this paper.

B. Patients and Methods

I. Patients

Eighteen patients entered in the study. They all had criteria of severe aplastic anemia [2, 3] defined as: Reticulocytes counts less than 20,000/μl, platelets counts less than 20,000/μl, granulocytes less than 500/μl and a deeply hypoplastic marrow. Their age ranged from 13 to 33 years (median 22 years). Seven patients were females and eleven were males. The etiology was post-hepatitic in two cases, benzol induced in two cases, paroxysmal nocturnal hemoglobinuria in aplastic phase in one case. No etiology was found in thirteen cases.

Before transplant, all patients had received more than 50 units of blood, none had received transfusions from a family member. Twelve patients had anti HLA antibodies and were refractory to random platelet transfusions.

II. Conditioning regimen

All patients received, on days -5 and -4,60 mg/kg/day of Cyclophosphamide. On day O they received total body irradiation (T.B.I.). Total body irradiation was given with a linear accelerator (Neptune, Thomson). The details of the technic have been described elsewhere [4].

The patient laid on his side and was rotated every 30 minutes to present the front, then the back to the source to assure the homogeneity of the dose. During the second half of irradiation, a lead brick wall was put in front of the thorax to shield the lung. Mediastinum was irradiated at the end of the session to give to this region the same dose as to the abdomen. During the long time irradiation, movements of the patient cannot be avoided. More over, there are large differences of thickness along the body. For these two reasons, we have prefered using "in vivo" measurement methods rather than calculations to determine the dose at different points of the patient. For this purpose three kinds of detectors were used: lithium fluoride thermoluminescent dosimeter, Therados Semiconductor probes and a Nuclear Entreprise ionization chamber.

Two patients received 1,000 cGy to the abdomen and 500 cGy to the lung. The next sixteen patients received 800 cGy to the abdomen and 400 cGy to the lung. The distance to the source varied during the study: five patients were at 5–6 meters from the source, the dose rate per minute varied from 4.6 to 5.8 cGy per minute, the length of irradiation from 3 hours 15 minutes to 6 hours and 40 minutes.

The other patients were irradiated at 4.2 meters from the source, the dose rate varied from 10 cGy to 12 cGy per minute, the length of irradiation varied from 2 hours and 30 minutes to 3 hours and 27 minutes. Table 1 gives details of the doses administered.

During irradiation, all patients received hyperdiuresis with glucose solution and electrolytes, phenobarbital 30 cg, antiemetics (métoclopamide), steroids if chills and fever were observed. They all received red blood cells and platelet transfusions before T.B.I. to maintain adequate counts during the procedure. The toxicity was generally mild: during the second half of irradiation fever, chills and nausea were observed. For 24 to 48 hours an erythematous rash, parotiditis and diarrhea were frequent and transient. There was no apparent delayed toxicity.

III. Bone Marrow Transplant procedure

1. Patients were isolated in reverse isolation rooms for at least two months. They received oral non absorbable anibiotics for gut decontamination, associated with a gluten free sterile diet. Skin, oropharynx, nasal, vaginal decontamination was attempted with antiseptic solutions. Nystatine was systematically given orally to prevent fungal infections.

All blood products received 2,500 cGy to avoid transfusions related G.V.H.D. Packed red blood cells and platelets transfusions were given prophylactically to maintain Hemoglobin levels above 8 g/100 ml and platelet counts above 20,000/μl. Granulocyte transfusions were given only when infection did not resolve with broad spectrum antibiotics within 24 hours.

2. The donor was an HLA identical sibling. In 11 cases donor and recipient were sex-matched, in 7 cases they were sex-mismatched. In 5 cases, a major ABO incompatibility between donor and recipient led to a plasmapheresis before bone marrow infusion. The patients received between 1.8×10^8 to 10×10^8 bone marrow cells per kilogram (median: 3×10^8 cells/kg). After grafting the patients received as a prevention of G.V.H.D., Methotrexate IV: 15 mg/sqm on day 1, then 10

Table 1. Dosimetry of T.B.I.

Patient No.	Abdomen dose cGy	Lung dose cGy	Dose Rate		Source distance m	Total length of Irradiation
			Mean cGy/mn	Instantaneous cGy/mn		
SL 18	1114	550	3.09	4.68	5.6	6 h 40
SL 28	950	540	3.02	4.85	5.6	5 h 15
SL 32	842	450	3.54	5.80	5.6	3 H 55
SL 33	782	489	3.40	5.48	5.6	3 h 50
SL 34	808	515	3.39	5.83	5.6	3 h 55
SL 36	773	402	4.17	10.1	4.2	3 h
SL 37	648	375	4.32	9.62	4.2	2 h 30
SL 38	782	362	3.78	8.98	4.2	3 h 27
SL 39	796	403	4.65	9.9	4.2	2 h 51
SL 40	834	415	5.63	10.46	4.2	2 h 30
SL 41	828	430	4.47	9.14	4.2	3 h 05
SL 42	781	404	4.5	11.9	4.2	2 h 50
SL 43	797	427	5.4	11.8	4.2	2 h 26
SL 45	805	430	4.85	12	4.2	2 h 46
SL 46	796	413	5	11.7	4.2	2 h 40
SL 47	808	399	4	11.7	4.2	3 h 22
SL 48	797	413	4.6	11.7	4.2	2 h 53
SL 49	790	388	5	11.7	4.2	2 h 39

mg/sqm on days 3, 6, 11, then weekly till approximately day 60. G.V.H.D., when severe, was treated either with high dose steroids or A.L.G. or both.

An I.V. hyperalimentation catheter was put before grafting and I.V. hyperalimentation was started as soon as the caloric intake was inadequate. Chronic G.V.H.D. was treated with steroids and Azathioprine.

C. Results

The results are summarized in Table 2. Eleven patients out of eighteen are currently alive with a follow-up ranging from 651 days to 48 days (median: 321 days). The actuarial survival curve shows 55% survival at 6 months. After this period a plateau is obtained (Fig. 1). A take was observed in all patients between day 9 to day 14. None of the patient rejected his graft. All have a complete hematologic reconstitution with chimerism as shown by the study of genetic markers (cytogenetic analysis, red blood cells antigens, Immunoglobulin allotypes).

Two patients (SL 39, SL 46) had an hypoplastic marrow on autopsy, both had severe G.V.H.D. treated with high doses antilymphocyte globulin and evidence of C.M.V. infection. One can assume that marrow hypoplasia was related to drug toxicity and viral infection as evidence of decreased marrow function occurred just before death in severely ill patients.

G.V.H.D. was observed in 16 out of 18 patients. In 4 patients, it was a transient skin rash which resolved spontaneously without further treatment. In

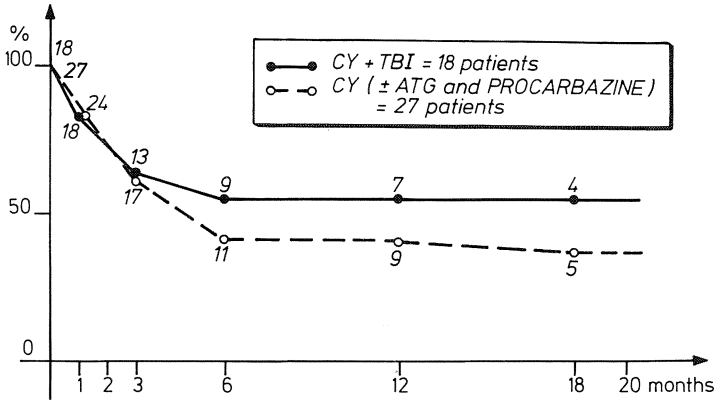


Fig. 1. Actuarial survival curve after B.M.T. in A.A.

Table 2. Evolution of B.M.T. after Cyclophosphamide and T.B.I.

Patient No.	Age/ Sex	Etiology	Anti-HLA Antibodies	Nb of cells/kg	Rejection	GVH	day	Survival days
SL 18	16/F	Idiopathic	++++	3.5×10^8	0	III	9	† 36 Infectious and metabolic complications
SL 28	22/M	Idiopathic	++++	3.3×10^8	0	III	7	> 651 Resolving chronic GVH
SL 32	22/F	Idiopathic	++++	10×10^8	0	III	22	> 479
SL 33	29/F	Idiopathic	++++	2×10^8	0	I	14	† 128 Subacute necrotizing hepatitis
SL 34	19/M	Post-hepatitis	−	2.6×10^8	0	I	9	> 461 Chronic hepatitis resolving
SL 36	15/M	Idiopathic	−	3×10^8	0	II	120	> 398
SL 37	22/F	Idiopathic	−	1.9×10^8	0	I	20	† 63 CMV interstitial pneumonitis
SL 38	13/M	Idiopathic	++++	3.5×10^8	0		0	> 350
SL 39	24/M	Post-hepatitis	++	1.6×10^8	0	III	28	† 63 GVH
SL 40	24/M	Idiopathic	−	1.8×10^8	0	III	50	> 321
SL 41	19/F	Idiopathic	−	2×10^8	0	I	15	> 259
SL 42	16/M	PNH Idiopathic	+++	2×10^8	0	III	20	† 50 Interstitial pneumonitis
SL 43	23/M	Idiopathic	+++	1.9×10^8	0	III	8	† 50 Interstitial pneumonitis + GVH
SL 45	22/M	Benzol	−	1.3×10^8	0		0	> 126
SL 46	20/M	Idiopathic	+++	3.3×10^8	0	III	20	† 74 GVH-Gut hoemorrages
SL 47	31/F	Idiopathic	++++	5×10^8	0	II	8	> 75 Doing well no GVH
SL 48	33/M	Benzol-PNH	++++	4.7×10^8	0	II	25	> 57
SL 49	16/F	Idiopathic	−	5×10^8	0	II	10	> 48

4 patients, a grade II G.V.H.D. with skin rash, mild diarrhea and liver enzymes rise was observed. It was treated with steroids. In three cases, it resolved completely. In one patient (SL 36) evidence of chronic G.V.H. marked by a lichen planus like eruption, chronic hepatitis and malabsorption necessitated a long term treatment with low doses steroids. This patient was still alive after more than 398 days and signs of chronic G.V.H.D. are slowly resolving while the dose of steroids in progressively diminished. Eight patients had severe grade III G.V.H.D. with diffuse erythemato-papular rash, hepatitis and profuse diarrhea. They were all treated with A.L.G. and steroids. Three patients survived with chronic G.V.H.D. currently treated with steroids and Azathioprine. One patient is normal on day 479 (SL 32), two patients have continuing skin lesions (sclero-atrophic lichen planus) (SL 28, SL 40), hepatic and gut abnormalities have disappeared. Despite persisting skin abnormalities they returned to normal activities. Their follow-up is 651 and 259 days. Five patients died as a consequence of G.V.H.D. Patient SL 18 died on day 36 of metabolic and infectious complications with active G.V.H.D. Patient SL 39 died of G.V.H.D. with gut hemorrhages, C.M.V. infection and low blood counts on day 63. Patient SL 42 died on day 50, skin lesions had completely resolved but hepatic and gut damage persisted with profuse bloody diarrhea. He died of interstitial pneumonitis possibly related to C.M.V. infection. Patient SL 43 died on day 52 of interstitial pneumonitis of unknown origin. At time of death, skin and gut lesions had resolved. Patient SL 46 died of intestinal hemorrhages on day 74. At time of death skin lesions had resolved but gut and liver G.V.H.D. persisted. It was associated with C.M.V. infection.

Two additional patients died with a good graft and no evidence of G.V.H.D. Patient SL 33 died on day 128 of viral subacute necrotizing non B hepatitis. Patient SL 37 died of C.M.V. interstitial pneumonitis on day 63. In summary, among the seven patients who died, G.V.H.D. was the primary cause of death in five cases, it was associated with interstitial pneumonitis in two cases, two patients died of viral infection without evidence of severe G.V.H.D. C.M.V. infection was observed in 12 cases. The diagnosis was assessed on a positive viremia, clinical evidence and late seroconversion. It was associated with interstitial pneumonitis and hepatitis in 6 cases. Seven patients survived to C.M.V. infection despite his association with G.V.H.D. Four patients had herpes zooster infection. It resolved simply in two cases, in the other cases it disseminated but finally was cured without further complications. It was treated with levamisole and hyperimmune globulin. Two patients had fungal infection, one an aspergillosus sepsis and one a candida sepsis. In both cases, it was completely cured by Amphotericin B. Bacterial infection was not a major problem and no patients died of sepsis.

D. Discussion

Bone marrow graft rejection has been the main cause of failure of transplant in severe aplastic anemia. The harmful effect of preceding transfusions has been confirmed by different authors [1, 7, 14, 18, 23, 24, 25]. In contrast, 24 untransfused patients showed sustained engraftment without rejection (Storb,

personnal communication). Storb et al. [16] analysed 72 consecutive patients with severe aplastic anemia. The multifactorial analysis showed that graft rejection correlated strongly with: 1. a positive relative response index in mixed leukocyte culture [10] and positivity of cell mediated immunity by the 51 Cr release test of patient against donor ($p<0.01$) and 2. a low number of marrow cells ($<3\times10^8$ cells/kg) used for transplantation ($p<0.05$).

Attempts have therefore been made to prevent marrow graft rejection in patients with positive in vitro tests of sensitization. 1000 rads T.B.I. in combination either with CYT 50 mg/kg on days -6, -5, -4 or with Procarbazine 15 mg/kg/day on days -8, -6, -5 and A.T.G. 15 mg IgG/kg on days -7, -5 and -3 were used. Eleven patients were treated with one of the T.B.I. regimen and only one rejected. Survival was poor, however, mainly because of death, secondary to interstitial pneumonitis, either associated with or following an episode of G.V.H.D. A second approach to reduce marrow graft rejection in sensitised patients has involved the infusion of un-irradiated buffy coat cells from the marrow donor on day 1, 2 and 3 following the infusion of bone marrow. This was done in the hope of infusing additional hemopoietic stem cells present among the peripheral leukocytes. Subsequently, 21 sensitized patients were given Cy and marrow plus 4×10^8 viable mononuclear blood cells/kg. This approach significantly reduced the rejection rate (15%) and increased survival (67%) [13].

Parkman et al. [11] found a presensitization against their sibling donors in 15 of 21 patients with severe aplastic anemia using the cell mediated lysis assay (CML). Twelve sensitized patients were transplanted after initial multiagent immunosuppression consisting of rabbit anti human thymocyte serum, procarbazine and cyclophosphamide. Engraftment was achieved in all of 11 patients who were evaluable and only two ultimately rejected their marrow grafts.

This study contrast with our own experience and the Seattle experience [21, 22] where no difference was found between CYT alone and A.T.G., PC and C.Y.T. Furthermore, we were not able to find any correlation in our previous study [7] between the number of marrow cells infused, R.R.I. positivity, the presence of anti HLA antibodies before grafting and marrow rejection [5]. For this reason and because all our patients had received more than 50 units of blood, we have treated all our patients with the same regimen using total body irradiation. T.B.I. has been widely used for conditionning patients with acute leukemia. Our protocol originated from the Seattle study on leukemics which used CYT 60 mg/kg for 2 days followed by 1000 rads T.B.I. [20, 22]. The overall survival was very poor. Mortality was related to G.V.H.D., interstitial pneumonitis and leukemic relapse. Interstitial pneumonitis has various causes, among them, the radiation damage may play a role. For this reason, we have decreased the total dose from 1000 rads to 800 rads and shielded the lungs which received only 400 rads. A careful dosimetry was performed during the whole procedure to control the homogeneity of the dose and the efficacity of shielding.

Our results show that this regimen has completely prevented bone marrow graft rejection as none of the patients rejected his graft. Three patients had lethal interstitial pneumonitis but this incidence is not significantly different from our previous experience with CYT where 4 out of 27 patients died with interstitial

pneumonitis. In contrast, it differs strongly with a previous series of leukemic patients who received at least 1000 rads on the lung and where 5 out of 10 patients died of interstitial pneumonitis. The immediate and delayed toxicity was therefore found mild but a longer follow-up is necessary to detect late toxicity such as cataracts or growth failure. Sterility is almost certain, but total azoospermia was also found in our male patients treated with Cytoxan two years after grafting.

The intensity of immunosuppression may increase the incidence and severity of G.V.H.D. Storb et al. [15] demonstrated that G.V.H.D. was correlated with 1. sex mismatch and 2. refractoriness to random platelet transfusions. Our series is too small to confirm this finding. The comparison with our previous and other teams' experience does not show a significant increase of the number of severe G.V.H.D.

Other authors have used different protocols of irradiation for aplastic anemia such as total lymphoïd irradiation [9] or low dose T.B.I. [6]. The results are encouraging despite the small number of patients treated.

Immunological tests, which predict with more accuracy the sensitization of the recipient against the donor, are strongly needed to separate patients who need Cytoxan alone from those who need a more intensive conditionning regimen.

References

1. Bortin, M., Rimm, A. Advisory committee of the Bone Marrow Transplant registry: Bone marrow transplantation from histocompatible donors for aplastic anemia. J.A.M.A. *236,* 1131–1135 (1976)
2. Camitta, B. M., Thomas, E. D., Nathan, D. G., Santos, G., Gordon Smith, E. C., Gale, R. P., Rappeport, J. M., Storb, R.: Severe aplastic anemia: a prospective study of the effect of early bone marrow transplantation on acute mortality. Blood *48,* 63–70 (1976)
3. Camitta, B. M., Rappeport, J. M., Parkman, R., Nathan, D. G.: Selection of patients for bone marrow transplantation in severe aplastic anemia. Blood *45,* 355–363 (1975)
4. Dutreix, A., Bridier, A.: Total body irradiation techniques and dosimetry. Pathol. Biol. (in press)
5. Elfenbein, G. J., Anderson, P. N., Klein, D. L., Schacter, B. Z., Santos, G. W.: Difficulties in predicting bone marrow graft rejection in patients with aplastic anemia. Transplant. Proc. *10,* 441–445 (1978)
6. Graze, P. R., Ho, W., Gale, R. P., for the UCLA bone marrow transplantation: Bone marrow transplantation for aplastic anemia conditioning with cyclophosphamide plus low dose total body irradiation. Exp. Hematol. *6,* Suppl. 3, 67 (1978) (abstract)
7. Gluckman, E., Devergie, A., Marty, M., Bussel, A., Rottembourg, J., Dausset, J., and Bernard, J.: Allogeneic bone marrow transplantation in aplastic anemia. Report of 25 cases. Transplant. Proc. *10,* 141–146 (1978)
8. Gluckman, E., Gluckman, J. C., Andersen, E., Devergie, A., Dausset, J.: Lymphocytotoxic antibodies and bone marrow grafts from HLA identical siblings. I HLA antibodies. Transplantation *26,* 284–286 (1978)
9. Kersey, J., Levitt, S., Ramsay, N., Krivit, M., Nesbit, N., Coccia, P., Tim, T.: Absence of rejection of human allogeneic marrow graft as a result of immunosuppression with total lymphoid irradiation T.L.I. and Cyclophosphamide. Exp. Hematol. *6,* suppl. 3, 37 (1978) (abstract)
10. Mickelson, E. M., Fefer, A., Storb, R., Thomas, E. D.: Correlation of the relative response index with marrow graft rejection in patients with aplastic anemia. Transplantation *22,* 294–300 (1976)
11. Parkman, R., Rappeport, J., Camitta, B., Levey, R. H., Nathan, D. G.: Successful use of multi agent immunosuppression in bone marrow transplantation of sensitized patients. Blood *52,* 1163–1169 (1978)

12. Santos, G. W., Sensenbrenner, L. L., Burke, P. J., Mullins, G. M., Anderson, P. N., Tutshka, P. H., Braine, H. G., Davis, T. E., Humphrey, R. L., Abeloff, M. D., Bias, W. B., Borgaonkar, D. S. and Slavin, R. E.: Allogeneic marrow grafts in man using Cyclophosphamide. Transplant. Proc. *6,* 345–348 (1974)
13. Storb, R., Thomas, E. D., Buckner, C. D., Fefer, A., Goodell, B., Neiman, P., Sanders, J., Singer, J., Weiden, P.: Progress in marrow transplantation for severe aplastic anemia. Blood *52,* Suppl. 1, 91 (1978) (abstract)
14. Storb, R., Thomas, E. D., for the Seattle marrow transplant team: Marrow transplantation for treatment of aplastic anemia. Clin. Haematol. *7,* 597–609 (1978)
15. Storb, R., Prentice, R. L., Thomas, E. D.: Treatment of aplastic anemia by marrow transplantation from HLA identical siblings. Prognosis factors associated with graft versus host disease and survival. The J.C.I. *59,* 625–632 (1977)
16. Storb, R., Prentice, R. L., Thomas, E. D.: Marrow transplantation for treatment of aplastic anemia. An analysis of factors associated with graft rejection. N. Engl. J. Med. *296,* 61–66 (1977)
17. Storb, R., Thomas, E. D. Weiden, P. L., Buckner, C. D., Clift, R. A., Fefer, A., Fernando, L. P., Giblett, E. R., Goodell, B. W., Johnson, F. L., Lerner, K. G., Neiman, P. E., Sanders, J. E.: Aplastic anemia treated by allogeneic bone marrow transplantation. Report of 49 new cases from Seattle. Blood *48,* 817–841 (1976)
18. Storb, R., Epstein, R. B., Rudolph, R. H., Thomas, E. D.: The effect of prior transfusion on marrow grafts between histocompatible canine siblings. J. Immunol. *107,* 409–413 (1970)
19. Thomas, E. D., and Storb, R.: Technique for human marrow grafting Blood. *36,* 507–515 (1970)
20. Thomas, E. D.: Bone marrow transplantation in acute leukemia. Lancet *I,* 859–876 (1978)
21. Thomas, E. D., Fefer, A., Buckner, C. D., Storb, R.: Current status of bone marrow transplantation for aplastic anemia and acute leukemia. Blood *49,* 671–681 (1977)
22. Thomas, E. D., Storb, R., Clift, R. A., Fefer, A., Johnson, F. L., Neiman, P. E., Lerner, K. G., Glucksberg, H., Buckner, C. D.: Bone marrow transplantation. N. Engl. J. Med. *292,* 832–843 (1975) first part, N. Engl. J. Med. *292,* 895–902 (1975) second part
23. Tutschka, P. J., Saral, R., Elfenbein, G. J., Sensenbrenner, L., Bias, W., Borgaonkar, D., Slavin, R. E., Santos, G. W.: Marrow transplantation in aplastic anemia – summary of Baltimore experience. Exp. Hematol. *5,* Suppl. 2., 46 (1977)
24. UCLA Bone Marrow Transplant Team: Bone marrow transplantation in severe aplastic anemia. Lancet *11,* 921–923 (1976)
25. Weiden, P. L., Storb, R., Slichter, S., Warren, R. P., Sale: Effect of six weekly transfusions on canine marrow grafts: tests for sensitization and abrogation of sensitization by Procarbazine and antithymocyte serum. J. Immunol. *117,* 143–150 (1976)

Discussion

Dicke: What is the age group and the overall survival of your leukemia patients?
Gluckman: None of them survived, they died of GVHD, leukemia relapse or no remission, the age ranged from 6 to 29 years. All were in the end stage of their disease.
Kersey: It is clear that you reduced the incidence of rejection by irradiation. To what extent is this due to a change in the preimmunisation status of your patients. Could this be demonstrated by in vitro tests?
Gluckman: All were heavily transfused so that we think that they were immunized. We have no test for preimmunization.
Tutschka: Are you treating all your patients with irradiation?
Gluckman: All our patients were preimmunized and therefore were irradiated. Of course, we would not irradiate patients who have not been transfused.
Tutschka: Our small series of patients with lung shielding also shows a reduction in the rate of interstitial pneumonitis.
Slavin: I would propose to use exact lead shields adapted for the lungs of each patient and to include the thymus into the irradiation field. This would give even better immunosuppression.
Gluckman: The thymus was irradiated at the end of the session.

Fliedner: We found after 800 R total body irradiation a sizeable amount of radiation induced chromosomal abnormalities in lymphocytes. This could give insight to the amount of remaining host T-cells. Have you observed that?
Gluckman: We have analysed the karyotypes in our grafted patients, also with PHA-stimulation, and not observed chromosomal abnormalities.
Lohrmann: You may be overtreating your patients, since the UCLA group has been successful with 300 to 350 rads total body irradiation and cyclophosphamide.
Gluckman: As far as I know there are only few patients in this study.
Storb: I think in both studies, the UCLA study and the Paris study observation time is too short for a final conclusion.
Lohrmann: What were the doses of steroids used in treatment of GVHD and the duration of treatment.
Gluckman: 2 mg per kg body weight as long as GVHD persisted.
Vriesendorp: The incidence of interstitial pneumonitis remained unchanged after lung shielding. Does not that mean that total body irradiation has nothing to do with interstitial pneumonitis.
Gluckman: I think that interstitial pneumonitis is caused by many factors one of them being damage by chemotherapy or irradiation, another infection.
Storb: I think, Dr. Vriesendorp's view is not unrealistic. In our 75–80 transplants from identical twins given 1000 rads without lung shidding and Cytoxan the incidence of interstitial pneumonia is about 5 per cent. In conclusion, other factors are more important.
Gluckman: you can not compare allogeneic and syngeneic transplants in this respect, since many other factors contribute in allogeneic situations.
Santos: I agree with Dr. Gluckman. In our leukemia patients treated with Cytoxan alone the incidence of interstitial pneumonia was the same as in aplastic patients which was the same as reported by the Seattle group. After starting total body irradiation we saw a significant increase in interstitial pneumonia.
Kersey: In support of Dr. Gluckman's finding we have seen no interstitial pneumonitis in our 11 patients with total lung shielding.
Storb: We have recently seen 30 leukemia patients in a row without interstitial pneumonia. So, I think that we are talking about too small numbers.
Dicke: I recall the paper of Dr. Golde and Dr. Thomas of replacement of lung macrophages in chimeras. I think radiation damages macrophages and stromal cells more than Cyclophosphamide. In autologous and syngeneic transplants you have a much faster regrowth of the macrophage population.

Role of Total Body Irradiation in Conditioning for Bone Marrow Transplantation

H. M. Vriesendorp* and D. W. van Bekkum

A. Introduction

The hemopoietic toxicity following accidental total body radiation can be circumvented by the injection of normal bone marrow (BM) cells after irradiation. The "hat-trick" of the protective effect of the injected BM cells appears to be the delivery of a new donor-type hemopoietic system [14, 25, 36]. After this was discovered, research efforts were reversed and unirradiated animals were deliberately irradiated to prepare ("condition") them to receive a new hemopoietic system. Radiomimetic drugs were later introduced as conditioning agents for BM transplantation [17, 18, 27]. Increasing numbers of patients with hemopoietic disorders such as acute leukemia, severe immunodeficiency (SCID) or aplastic anemia (AA) appear to benefit from BM transplantation [2, 3, 34]. Optimal conditioning regimens for human BM transplantation remain to be defined. In this paper the information on total body irradiation (TBI) as a conditioning agent in experimental animals will be reviewed. On this data base new suggestions for the use of TBI in human BM transplantation will be made.

B. Aims of Conditioning

The three human diseases that have been treated with BM transplantation most frequently can be used to illustrate the principles of conditioning (see Table 1). A pivotal role is played by the transplanted hemopoietic stem cells (HSC). This cell population must "take" in the new host and expand through self-replication to secure complete hemopoietic regeneration. HSC are also responsible for the production of donor end cells (erythrocytes, thrombocytes, granulocytes, monocytes and lymphocytes) by differentiation and maturation processes. Replication and differentiation of HSC will occur only if the proper "space" is available. The first aim of conditioning will be to create sufficient space for the incoming HSC's. More detailed information on the nature or anatomical location of space is lacking. Histological examination of BM biopsies indicate that space would not be available for allogeneic HSC in the SCID patients, except perhaps in the thymus. This might explain why, in these patients without any conditioning,

* This investigation was supported in part by a Public Health Service International Research Fellowship (number Fo5 TW-2562)

Table 1. Conditioning for bone marrow transplantation

	Severe combined immune deficiency	Aplastic anemia	Acute leukemia
Space HSC	no	yes	no
Resistance HSC	no	yes	yes
Tumor cells	no	no	yes
Aims of conditioning	none space?	↓resistance	↑space ↓resistance ↓tumour cells

T lymphocytes carrying donor type markers, emerge after BM transplantation, while other hemopoietic end cells continue to show recipient types (Niethammer et al., this volume). In AA patients, sufficient space would appear to be present in the BM, whereas this is generally not the case in leukemic patients.

Lasting functional activity of the donor HSC can occur only, if rejection of these cells by the recipient can be prevented. The tendency of the recipient to inactivate allogeneic HSC has been designated resistance in preference to the term rejection. Results of studies in mice suggest that the two phenomena are different in time of occurrence after transplantation, in development in neonatal individuals and in sensitivity to radiation and antimacrophage agents [11]. Resistance does not take place in SCID patients, because of the absence of a functioning immune system. Resistance is manifested in AA and leukemic patients and is increased in comparison to normal individuals as a result of sensitization against HSC antigens through cross-reactive antigens contained in transfusions given prior to the BM transplant.

A third aim of conditioning is only relevant in patients with leukemia. The replacement of the malignant hemopoietic system by a new normal hemopoietic system will be of lasting usefulness only, if all clonogenic leukemic cells in the host can be eliminated. Therefore, conditioning agents should also lead to a pronounced and, if possible, definitive eradication of the leukemic cells of the patient.

C. Properties of Currently Known Conditioning Agents

I. Space

When animals appear to die after high dose chemo- or radiotherapy with signs of hematological insufficiencies in toxocological research, it is logical to attempt to protect the animals from this toxicity by an infusion of autologous bone marrow cells harvested before the insult. If lethality can be prevented by this procedure, it demonstrates that HSC toxicity is dose limiting at the investigated drug dose level. Moreover, it shows that sufficient space for the transfused HSC's was available. Resistance can be measured independently of space by comparing the results obtained in autologous rescue experiments to those of similar tests with

Table 2. Rescue with autologous BM cells after supralethal chemo/radiotherapy[a]

Agent	Dose kg^{-1}	Autologous BM cells $.kg^{-1}.10^{-8}$	Fraction surviving dogs	Dose limiting toxicity	Author
ThioTEPA	6 mg i.v.	?[b]	3/3	GI	Lochte et al. 1963 [21]
CCNU	20 mg i.v.	3	4/4	GI	Abb et al. 1978 [1]
DMM	10 mg i.v.	±5	7/7	GI	Kolb et al. 1974 [19]
CY	100 mg i.v.	±3[b]	8/10		Epstein et al.
	40 mg i.v. days −3, −2, −1	7	9/13	GI bladder	1969 [13] Kolb et al. 1976 [20]
TBI	750 rad	.5	4/4	GI	Vriesendorp et al. 1976 [40]

[a] ThioTEPA = Triethylenethiophosphoramide; CCNU = 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosurea; DMM = Dimethylmyleran; Cy = Cyclophosphamide; TBI = Total Body Irradiation; GI = Gastrointestinal

[b] Cryopreserved BM

allogeneic BM. A summary of the results of autologous BM rescue experiments after high dose chemo- or radiotherapy in dogs is given in Table 2. All agents in Table 2 appear to create sufficient space. Similar results have been obtained in other species [4, 6]. With autologous BM marrow transplantation, higher doses of conditioning agents can be given and gastrointestinal toxicity becomes dose limiting in all cases. L-asparaginase (L-Asp) and Antilymphocyte Serum (ALS) are conditioning agents with other dose limiting toxicities. They have not received much attention as single agents in conditioning, presumably because they do not create sufficient space. However, differences in dose limiting toxicity of conditioning agents will decrease the toxicity of multiple agent conditioning protocols. Several combinations between ALS or L-Asp and the agents of Table 2 have been shown to be effective in preparing recipients for allogeneic BM cells [32, 39].

II. Resistance

Unfortunately, no prospective in vitro test is presently available to measure resistance [40] in unimmunized recipients. Resistance can only be quantified in vivo by determining the ratio between the number of allogeneic and autologous BM cells required for hemopoietic recovery in 50% of the unmodified animals. Resistance can be increased in recipients, which have been immunized by blood transfusions prior to BM transplantation. Recently, it was demonstrated that the presence of this increase in resistance can be predicted by cocultures of donor bone marrow cells and recipient lymphocytes [35]. In this paper, resistance will be evaluated for unsensitized individuals only. The effect of TBI on resistance is

Table 3. Relative bone marrow dose required for 50% radioprotection after supralethal TBI[a]

BM donor	Mice	Dogs	Rhesus monkeys
Autologous/isologous	1	1	1
MHC[b] identical	4	4	n.d.
MHC[b] mismatched	60	±60	±40

[a] Total Body Irradiation ± 800 rad X-ray in all species
[b] MHC = Major Histocompatibility Complex

given in Table 3. After supralethal TBI, considerable resistance remains against BM cells of a donor identical for the major histocompatibility complex (MHC). Four times more cells are required from such a donor than from an autologous or isologous source. Tremendous resistance against MHC mismatched BM cells remains under those conditions, since 40 to 60 times more BM cells appear to be needed for rescue. Results of single agent conditioning in dogs with Dimethylmyeleran (DMM), Cyclophosphamide (CY) and TBI are given in Table 4. Bone marrow cell titrations have not been performed for DMM and CY. In the summarized experiments, BM cells and peripheral blood leukocytes (PBL) were given i.v. a dose of 4 to $7 \times 10^8.kg^{-1}$ and 1.3 to $3.3 \times 10^9.kg^{-1}$, respectively. Conditioning with DMM and CY appears to lead to split chimerism in MHC identical donor recipient pairs. This might be due to the relatively minor effects of these agents on host resistance. In contrast, TBI appears to be the most effective conditioning agent in MHC identical pairs. Complete chimerism is obtained in almost all animals. The addition of PBL to BM cells has been shown to decrease allogeneic resistance [31]. With this procedure, lasting takes are observed in all animals after CY but not after DMM conditioning in MHC identical combinations.

In MHC mismatched donor recipient pairs, takes can be obtained if very high numbers of BM cells are given or if moderate numbers of BM cells are given in

Table 4. Allogeneic resistance after single agent conditioning[a]

Agent	Dose	MHC donor ≠	Fraction of dogs with take	MHC donor =	Fraction of dogs with take	Chimerism	Author
DMM	10 mg i.v. kg^{-1}	BM+PBL	0/4	BM+PBL	8/16	split	Kolb et al., 1974 [19]; Storb et al., 1977 [32]
CY	120 mg i.v. kg^{-1}	BM	1/4	BM	6/9	split	Rieder et al., 1978 [26]
	day −3, −2, −1	BM+PBL	3/6	BM+PBL	9/9	split	
TBI	750 rad	BM	0/6	BM	24/24	complete	Vriesendorp et al., 1975 [38]
		BM+PBL	9/11	BM	10/11	complete	Weiden et al., 1977 [42]

[a] DMM = Dimethylmyleran; CY = Cyclophosphamide; TBI = Total body irradiation; BM = Bone marrow; PBL = Peripheral blood leukocytes; = Identical; ≠ Mismatched

combination with PBL. Again, TBI appears to be the most effective single conditioning agent with moderate activity of CY and no activity of DMM.

III. Tumor cell kill

The number of tumor cells killed by conditioning agents will depend on the sensitivity of the tumor cells to the drug/radiation dose to which they are exposed. TBI has the advantage that it will kill noncycling tumor cells and that no pharmacological sanctuaries exist for this agent. A disadvantage of TBI is that the number of decades of tumor cells that will be killed by the maximum acute TBI dose (±800 rad, 300 kV X-rays or its equivalent) is limited. When the Do's (70–300 rad) found for various tumor cell lines are taken into account, this amounts to a maximum of approximately four decades [43]. In this respect, CY and DMM might be more effective than TBI. For CY and Busulphan, this has been shown in a rat acute myeloid leukemia model [28; Hagenbeek, unpublished observations]. Further quantitative studies in animal models predictive for responses in human cancer patients will be required for the selection of conditioning agents with maximum tumoricidal activity in leukemic patients or other oncological patients for which a treatment protocol including BM transplantation is considered.

IV. Role of TBI

A summary of single agent conditioning is given in Table 5. It appears that none of the currently known agents is capable of fulfilling all the goals of conditioning. TBI appears to be most useful in suppressing resistance. In cancer patients and in patients without an MHC identical donor, additional conditioning will be required. In multiple agent conditioning, it would be advantageous to apply the same principles as developed for high dose multiple agent chemotherapy [16], that is, to combine active agents with different mechanisms of action and

Table 5. Summary single agent conditioning[a]

		TBI	CY	DMM
Creation of space		++	++	++
Abrogation of resistance	MHC =	++	+	±
	MHC ≠	+	±	−
Tumour cell kill	Go cells	+	±	±
	sanctuaries	+	−	−
	decades	2–4	6[b]	2–4[b]
Dose limiting toxicity		GI	GI bladder	GI

[a] TBI = Total body irradiation; CY = Cyclophosphamide; DMM = Dimethylmyleran; MHC = Major histocompatibility complex; GI = Gastrointestinal

[b] Sharkis and Santos, 1977 [28]; Hagenbeek and Martens, 1979 (unpublished observations). Rat myeloid leukemia model

nonoverlapping dose limiting toxicity. In the following sections of this paper, the optimal use of TBI is further defined to facilitate its inclusion in multiple agent conditioning protocols in man.

D. Number of Bone Marrow Cells Required for Radioprotection

I. Man

Obvious ethical constraints prohibit the direct determination of the LD50 for TBI in man or the number of BM cells required to rescue human patients from a lethal exposure to ionizing radiation or chemotherapy. Therefore, an indirect approach, that of extrapolating the data obtained in experimental animals to man, has to be used. Table 6 lists the LD50 for TBI and the BM cell dose required for 50% rescue after supralethal TBI in four mammalian species (mouse, rat, rhesus monkey and dog) as determined in our laboratory [4, 10, 38; Vriesendorp and van Bekkum, unpublished observations]. As previously noted by others [9], the larger species appear to have a lower LD50 for TBI. In addition, they require more BM cells for rescue after a supralethal TBI dose. In Figure 1, a linear regression is shown between body weight and the 50% BM cell rescue dose. There is a significant positive correlation between the two ($r=0.99$, $p=0.009$). The extrapolated BM rescue dose for a human being weighing 70 kilograms is also shown. Doubling the 50% BM rescue dose in animals gave a 100% rescue dose in all species. Thus when this 50% human rescue dose is doubled, an estimate for a 100% rescue dose in man of $4\times10^7.kg^{-1}$ is obtained.

II. Species differences

Theoretically, three possible explanations can be offered for the differences observed among species in LD50 for TBI and BM rescue dose:
1. differences in radiosensitivity of HSC among species;
2. differences in kinetic parameters of the hemopoietic system in the different species;
3. differences in the number of HSC per body unit (kg body weight or m^2 surface area) among species.

Two reasonable assumptions have to be made to demonstrate that the first explanation is probably incorrect: 1. The fraction of total bone marrow required for radioprotection has to be equal to the fraction of total HSC required for radioprotection; and 2. the fraction of HSC surviving an LD50 TBI has to be equal to the fraction of HSC which seeds ("homes") in the bone marrow and is responsible for 50% radioprotection after supralethal TBI. From Table 7, it can be seen that the fraction of total bone marrow (fraction HSC) surviving an LD50 in mice is approximately 10^{-5}. To reach this survival level at 700 rad, a Do of approximately 65 rad would be required (if $n=1$). This is close to the Do value reported for CFU-S in this species [7]. However, if only differences in radiosensitivity of HSC's could explain the differences listed in Table 6, an unrealistic low Do of approximately 35 rad would have to be postulated for the

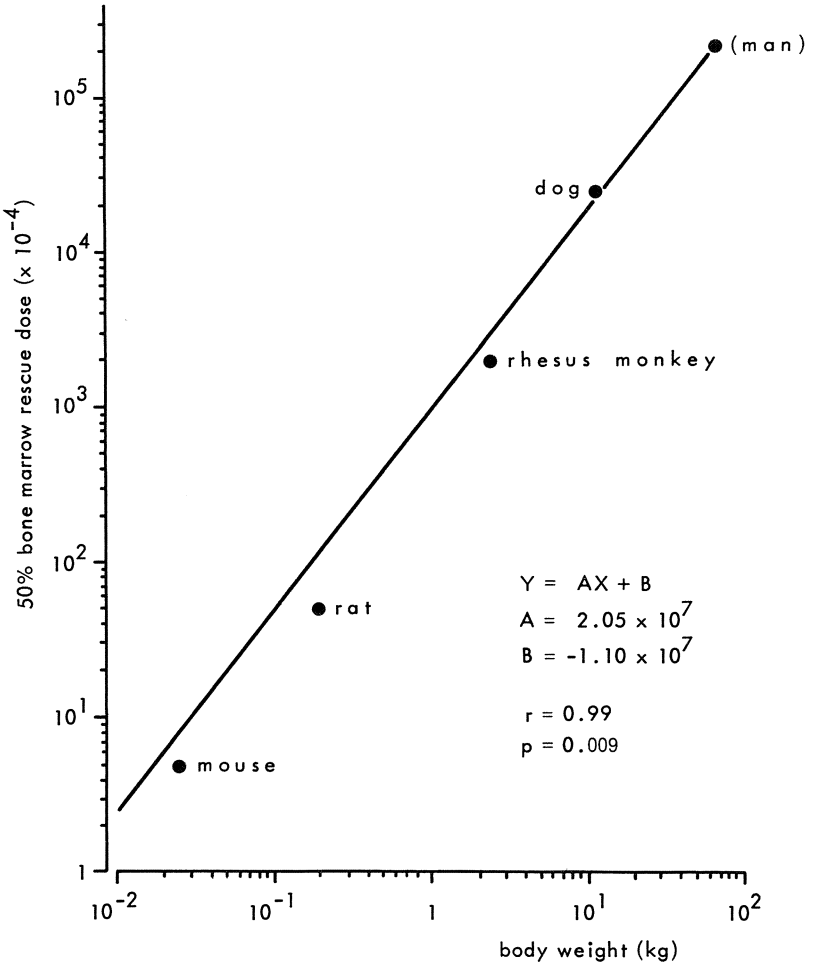


Fig. 1. Correlation between body weight and bone marrow dose for 50% rescue after supralethal total body irradiation

HSC in the most radiosensitive species – the dog – to obtain the same fractional HSC survival of 10^{-5} at the LD50 TBI dose in this species. Another argument against this explanation is that radiosensitive species require more HSC's for rescue after supralethal TBI. The postulated extreme radiobiological differences would not explain the observed differences in requirements for fresh unirradiated HSC.

The second explanation would hold under a particular combination of differences in kinetic properties of the hemopoietic system. Larger species would require more BM cells for rescue when their marrow transit time (i.e., the time needed by a HSC to deliver an end cell) would be longer for end cells which are critical for survival. In addition, a shorter survival time of these end cells has to be postulated in the larger species. This would lead to lower LD50 TBI doses in such

Table 6. LD50 TBI and minimum BM cell dose for radioprotection after LD 100 TBI

Species[a]	LD50 in rad[b]	BM rescue dose $\times 10^{-6}$[c]	
		Per kg body weight	Per m^2 BSA
Mouse	700	2.0	6.5
Rat	675	3.8	24.2
Rhesus monkey	525	7.5	80.0
Dog	370	17.5	445.0

[a] Acidified drinking water for all animals. Platelets, blood transfusions and antibodies in monkeys and dogs only. Endpoint 30 day survival in all species.
[b] Ten or more animals per point for mice and rats; four or more animals per point for monkeys and dogs. Intervals between TBI doses tested, 100 rad.
[c] BM doses tested differed by a factor 2

species. Both postulates can be shown to be incorrect, since the kinetics of the disappearance of granulocytes and thrombocytes after TBI and the regeneration of these cells after a successful low dose BM transplant are similar in all species.

The preceding elimination of explanations 1 and 2 makes the third one the most likely. Different arguments in favour of this explanation can be given. 1. It is reasonable to assume that the same minimum number of active HSC per body unit is required for protection against the BM syndrome after TBI in all species. More radiation will be required to reach this minimum critical level in a species with a high initial concentration of HSC and fewer BM cells will make up this critical number in rescue experiments. This can explain the negative correlation observed between LD50 for TBI and BM rescue dose. 2. In this explanation, the

Table 7. Estimation of fraction of bone marrow required for survival after supralethal TBI

Species (body weight in kg)	(1)[a] Total number of bone marrow cells per species	(2) Total number of injected bone marrow cells for 50% rescue after supralethal TBI	(3) Fraction of total number of bone marrow cells required for 50% survival after supralethal TBI[b]
Mouse (.025)	1.0×10^9	5×10^4	1.2×10^{-5}
Rat (.2)	5.9×10^9	50×10^4	3.1×10^{-5}
Rhesus monkey (2.6)	62.4×10^9	195×10^5	1.2×10^{-4}
Dog (12.0)	144×10^9	240×10^6	5.5×10^{-4}

[a] $(3) = \dfrac{\text{(2) homing fraction } (1/3)^{c} + \text{fraction bone marrow cells surviving supralethal total body irradiation } (Do = 60 \text{ rad}, n = 1)}{(1)}$

[b] Pegg, 1966, with the exception of the dog [24]
[c] van Bekkum et al., 1978 [8]

assumption is made that HSC's in the various species differ in concentration but have the same Do. The fractional survival of HSC at the LD50 TBI dose for the different species as given in Table 7 can be used to estimate this Do. Such an analysis is shown in Figure 2 and predicts a Do of between 50 and 75 rad for HSC. The significant correlation ($r=0.99$, and $p<0.001$) in a logarithmetic regression of the points shown in Figure 2 indicates that the assumed interconnections

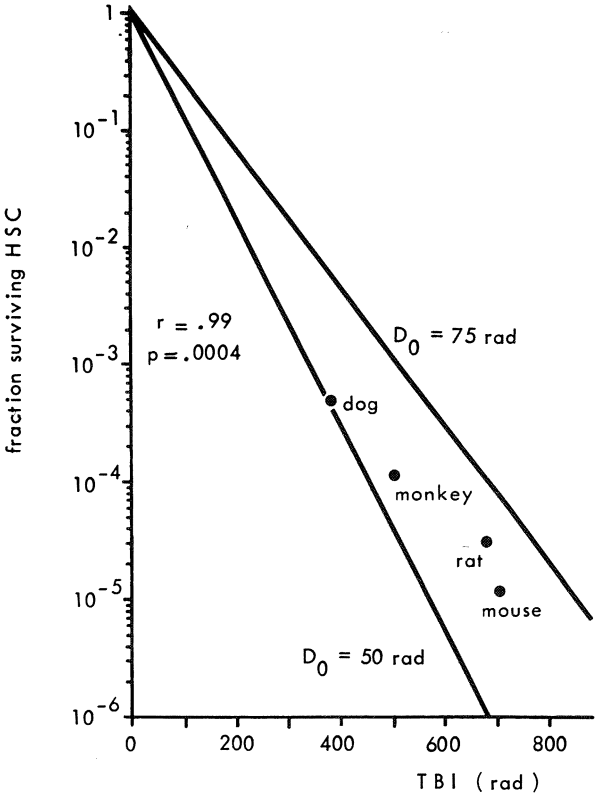


Fig. 2. Estimation of radiation survival of hemopoietic stem cells

between the points are not unrealistic. 3. Another implication of the model is that the fraction of the total bone marrow required for 50% rescue not only reflects the concentration of HSC in that species but also allows a prediction of the LD50 TBI dose. Species which require a high fraction of their total bone marrow for rescue after supralethal TBI will have a low LD50 for TBI. The estimated BM rescue dose for man (see C 1) divided by the total BM dose in this species [24] can be fitted to the curve shown in Figure 2 and predicts a LD50 for TBI in man of 315 rad kV X-ray. This is close to the estimate of 300 rad based on radiation accidents [23].

E. Optimal TBI Dose for Conditioning

The minimum TBI dose required for the maximum suppression of resistance against an MHC identical transplant was determined in dogs. Such experiments are best done in this species because of the availability of MHC identical donor-recipient pairs and an HSC concentration close to that expected in man (see section C) in this experimental animal model.

Table 8. TBI dose and MHC identical BM transplants[a]

TBI rad	Number of dogs tested	% complete chimeras 100 days post TBI
750 (supralethal)	20	20/20
500 (±LD100)	5	5/5
350 (±LD50)	5	0/5

[a] 300 kV X-ray – dose rate 18 rad per minute 10 mA, HVL 3 mm Cu, 4×10^8 BM cells. kg^{-1}

The results of MHC identical BM transplants after 750, 500 and 350 rad TBI are shown in Table 8. After a low BM cell dose, a dosage of 500 rad appears to be sufficient to obtain a complete radiation chimera. The completeness of the chimerism was ascertained by sex chromosomes, polymorphic erythrocyte and

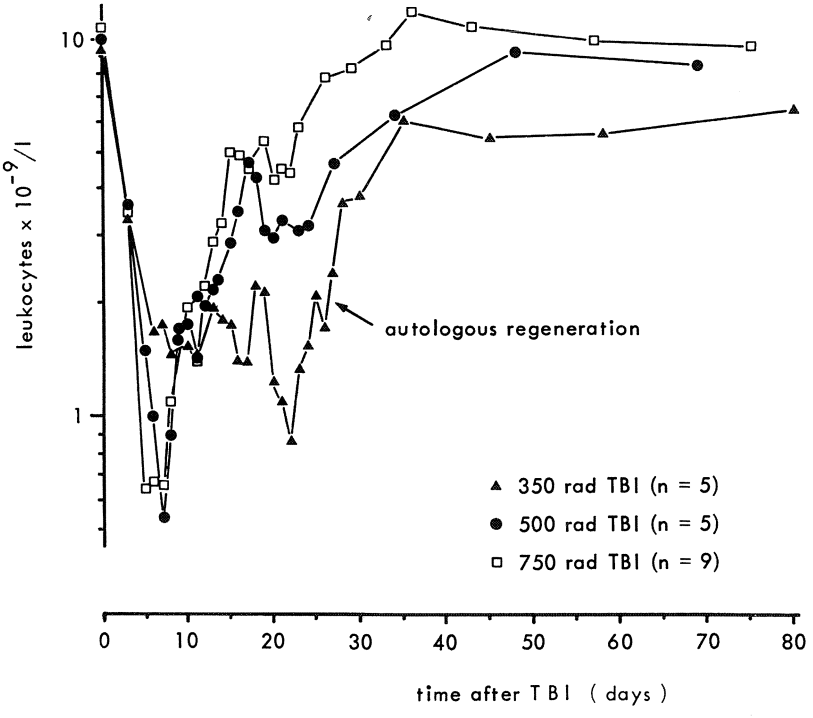


Fig. 3. Peripheral leukocytes after TBI followed by 4×10^8 DLA identical BM cells .kg $^{-1}$

leukocyte enzymes [38] and the survival of a kidney graft of the BM donor. After 350 rad, initial hemopoietic recovery is curtailed by the recipient's resistance. One out of the five animals given this treatment died during the subsequent aplastic phase. The remaining four survived through recovery of their own hemopoietic system. The mean leukocyte levels of the animals summarized in Table 8 after TBI are shown in Figure 3. After 350 rad, the inactivation of the allogeneic HSC's appears to be complete in approximately 20 days. This is similar to the rejection time of DLA skin allografts in unmodified recipients [37]. It differs from the concept of allogeneic resistance against HSC's as developed in mice, where the inactivation of allogeneic HSC's is presumed to take place within 48 hrs after injection [10]. The recipient's HSC's are eliminated by TBI through first order kinetics. After 750 and 500 rad, a considerable portion of recipient's HSC's will remain intact. The observation that complete chimerism is obtained, notwithstanding this surviving fraction of recipient's HSC's, can be explained by the elimination of this surviving cell population through GvH reactions. HSC's are known to be targets for GvH reactions [2, 4, 33]. One could speculate that the dips in the leukocyte curves in Figure 3 around day 18 after 750 and 500 rad TBI conditioning reflect the "war" between the donor's and recipient's hemopoietic systems. The second, continuing, rise of leukocyte levels after day 23 in these animals could indicate that the donor has won the war. A TBI dose of 500 rad is obviously the optimal TBI dose, because it gives the desired end result – complete donor hemopoiesis – at the lowest toxicity cost. The usefulness of low dose TBI conditioning in recipients sensitized by preceding blood transfusions remains to be determined and is currently under investigation in the dog model.

F. Recommendations for Human BM Transplantation

I. TBI dose

Significantly less gastrointestinal toxicity is observed after 500 rad TBI in dogs in comparison to 750 rad. Also thrombocytopenia and endogeneous infections occur significantly later (1 and 2 days, respectively) after the lower TBI dose [Vriesendorp, unpublished observations]. Dogs, female as well as male, regain their fertility within a year after 500 or 750 rad TBI. The sex ratio and the health of their offspring appear to be normal [Vriesendorp, unpublished observations]. In monkeys, a lower cataract incidence and no skeletal growth inhibition were observed after 500 rad TBI [29, 30]. Radiation pneumonitis is not seen in man below 600 rad TBI [15]. The lower host toxicity of 500 rad could be very advantageous in further attempts in multiple agent conditioning where an increase in toxicity can be anticipated by the addition of other agents to TBI.

Fractionated TBI, shielding of organs limiting the TBI dose or low dose rate TBI could possibly further decrease the toxicity of TBI. However, these procedures have not been shown to lead to an increased effect on tumor cells or host resistance simultaneously with a decreased or equal toxicity to normal tissues [4, 6; Hagenbeek, unpublished observations]. Shielding procedures are contraindicated in tumor patients, because of the risks of shielding tumor cells. In contrast,

an acute high dose rate exposure has the advantage of short patient treatment times (<1 hour) and a short interval between conditioning and bone marrow cell infusion. A disadvantage of a lower TBI dose is a decrease in tumor cell kill by 1 to 2 decades. However, TBI alone will never be sufficient for complete tumor eradication. A lower TBI dose with lower host toxicity will offer better possibilities for tumor eradication conditioning schemes incorporating additional, more effective agents.

II. BM cell dose

The extrapolated BM cell numbers .kg^{-1} required for transplantation after TBI conditioning for a human autologous, MHC identical or MHC mismatched donor are respectively, 4×10^7, 1.6×10^8 and 2×10^9 (see Table 3 and Fig. 1). The latter number is of course prohibitively high. The MHC identical and autologous donor BM dose recommendations are smaller than the ones currently in use and previously recommended [5]. An advantage of the smaller BM dose is that it increases the applicability of preparative BM procedures at which cell losses occur such as gradient separation [12] or incubation with antilymphocyte globulin [22]. These measures attempt to reduce the severity of GvH reactions by elimination of the post-thymic T cells in the graft [6]. A smaller dose of unpurified BM cells will decrease the incidence and severity of GvH disease if BM cells are contaminated with substantial numbers of postthymic T cells, as reported for primate BM [6]. This expectation is borne out by observations in mice and dogs, where the lethality and severity of GvH correlated with the number of lymphocytes transplanted [4, 41]. Indeed, a retrospective analysis of transplanted leukemic patients in Seattle shows a significantly higher GvH incidence in patients with myeloid leukemia who received more than 2×10^8 BM cells.kg^{-1}. However, this is not found in patients with lymphatic leukemia (34). In the same series of patients, graft failures appear to be significantly higher in patients receiving less than 1×10^8 BM cells (2 out of 7) when compared to patients receiving higher BM cell numbers (5 out of 90). Retrospectively, this result appears to fit the model sescribed in section D, which predicts an increasing graft failure rate in man when less than 8×10^7 BM cells.kg^{-1} are transplanted.

A smaller BM cell dose will result in a slower BM regeneration after conditioning and might increase hematological and bacteriological problems due to a longer BM aplasia after transplantation. However, a considerable portion of these problems is probably related to GvH disease and, if indeed a better GvH prevention is obtained by a smaller BM dose, in fact a decrease in these problems might be found.

G. Conclusions

None of the currently used conditioning agents appears to be sufficiently active to achieve all aims of conditioning as a single agent, i.e.: 1. the creation of space for transfused hemopoietic stem cells; 2. the depression of allogeneic resistance; and 3. the eradication of tumour cells. Multiple agent conditioning remains to be

developed. Total body irradiation (TBI) will play an important role in such conditioning regimens, since it creates sufficient space, effectively depresses the resistance against major histocompatibility complex (MHC) identical bone marrow (BM) cells and kills Go tumour cells as well as tumour cells in sanctuaries. Experimental studies in mice, rats, rhesus monkeys and dogs indicate that lower concentrations of hemopoietic stem cells are found in species with a heavier body weight and that maximum dose TBI is not required for a BM transplant from an MHC identical donor. Host HSC's which survive the TBI conditioning will be eliminated by GvH reactions if a sufficient TBI dose and sufficient BM cell numbers are given. Extrapolations to man indicate that, theoretically, a lower TBI dose and smaller BM cell numbers than currently used in human BM transplantation could be applied. The advantages of such an approach include: 1. lower host toxicity; 2. better applicability of in vitro preparative procedures for BM cell suspensions; and 3. a decreased incidence and severity of GvH reactions. Disadvantages could be a temporary increase in hematological and infectious complications through a prolonged period of aplasia immediately after conditioning and a higher fraction of surviving tumor cells in cancer patients. Additional measures can be taken to overcome these disadvantages. The possible advantages seem to outweigh these drawbacks and trials in human BM transplantation incorporating a lower BM cell dose and a smaller TBI dose appear to be justified.

Acknowledgements

The technical assistance of Trudi Visser, Wim Klapwijk, Barbara Brown and Albert Brivkalns is gratefully acknowledged. During these studies, helpful discussions with Drs. B. A. R. Ponder, L. Parker and R. Yankee led to the gathering of many ideas. Their assistance in subsequently eliminating most of them is highly appreciated.

References

1. Abb, J., Netzel, B., Rodt, H. V., Thierfelder, S.: Autologous bone marrow grafts in dogs treated with lethal doses of 1-(2-chloroethyl)-3-cyclohexy-1-nitro-urea. Cancer Res. *38*, 2157 (1978)
2. Advisory Committee of the International Bone Marrow Transplant Registry: Severe combined immunodeficiency disease. Characterization of the disease and results of transplantation. JAMA *238*, 591 (1976)
3. Advisory Committee of the International Bone Marrow Transplant Registry: Bone marrow transplantation from histocompatible, allogeneic donors for aplastic anemia. JAMA *236*, 1131 (1976)
4. Bekkum, D. W., van, Vries, M. J. de: Radiation chimeras. London: Logos Press, Academic Press 1967
5. Bekkum, D. W., van: Hostile grafts. In: Dausset, J., Hamburger, J., Mathé, G. (Eds.): Advance in transplantation, p. 565. Copenhagen: Munksgaard 1968
6. Bekkum, D. W., van: The double barrier in bone marrow transplantation. Semin. Hematol. *11*, 325 (1974)
7. Bekkum, D. W., van, Schotman, E. M.: Protection from hemopoietic death by shielding versus grafting of bone marrow. Int. J. Radiat. Biol. *4*, 361 (1974)
8. Bekkum, D. W., van, Löwenberg, B., Vriesendorp, H. M.: Bone marrow transplantation. In: Hirsch, D. W. (Ed.): Immunological engineering, p. 179. Lancaster: MTP Press Ltd. 1978

9. Bond, V. P., Robertson, J. S.: Vertebrate radiobiology (lethal actions and associated effects). Ann. Rev. Nuclear Sci. *7,* 135 (1957)
10. Broerse, J. J., Bekkum, D. W., van, Hollander, C. F., Davids, J. A. G.: Mortality of monkeys after exposure to fission neutrons and the effect of autologous bone marrow transplantation. Int. J. Radiat. Biol. *34,* 253 (1978)
11. Cudkowicz, G., Bennett, M.: Peculiar immunobiology of bone marrow allografts. I. Graft rejection by irradiated responder mice. J. Exp. Med. *134,* 83 (1971)
12. Dicke, K. A., Bekkum, D. W., van: Allogeneic bone marrow transplantation after elimination of immunocompetent cells by means of density gradient centrifugation. Transplant. Proc. *3,* 666 (1971)
13. Epstein, R. B., Storb, R., Clift, R. A., Thomas, E. D.: Autologous bone marrow grafts in dogs treated with lethal doses of cyclophosphamide. Cancer Res. *29,* 1072 (1969)
14. Ford, C. E., Hamerton, J. L., Barnes, D. W. H., Loulit, J. F.: Cytological identification of radiation chimeras. Nature *177,* 452 (1963)
15. Fowler, J. F., Travis, E. L.: The radiation pneumonitis syndrome in half-body radiation therapy. Int. J. Radiat. Oncol. Biol. Phys. *4,* 1111 (1978)
16. Frei, E. III: Combination cancer therapy. Presidential Address. Cancer Res. *32,* 2593 (1972)
17. Gale, R. P., Feif, S., Opelz, G., Territo, I., Young, L., Sarna, G., Fahey, J., Cline, M. J.: Bone marrow transplantation in acute leukemia using intensive chemoradiotherapy (SCARI-UCLA). Transplant. Proc. *8,* 611 (1976)
18. Graw, R. G., Lohrmann, H. P., Bull, M. I., Decter, J., Herzig, G. P., Bull, J. M., Leventhal, B. G., Yankee, R. A., Herzig, R. H., Krueger, G. R. F., Bleyer, W. A., Buja, M. C., McGinniss, M. H., Alter, H. J., Whang Peng, J., Gralnick, H. R., Kirkpatrick, C. H., Henderson, H. S.: Bone marrow transplantation following combination chemotherapy immunosuppression (BACT) in patients with acute leukemia. Transplant. Proc. *6,* 349 (1974)
19. Kolb, H. J., Storb, R., Weiden, P. L., Ochs, H. D., Kolb, H., Graham, T. C., Floersheim, G. L., Thomas, E. D.: Immunologic, toxicological and marrow transplantation studies in dogs given dimethylmyleran. Biomedicine *20,* 341 (1974)
20. Kolb, H. J., Rieder, I., Grosse-Wilde, H., Abb, J., Albert, E. D., Kolb, H., Schäffer, E., Thierfelder, S.: Marrow grafts in LD-SD typed dogs treated with cyclophosphamide. Transplant. Proc. *8,* 555 (1976)
21. Lochte, H. L., Kasakura, S., Karetzky, M., Ferrebee, J. W., Thomas, E. D.: Infusion of marrow in the mouse and dog after Thio-TEPA. Blood *21,* 424 (1963)
22. Müller-Ruchholtz, W., Woltge, H. U., Müller-Hermelink, H. K.: Bone marrow transplantation in rats across strong histocompatibility barriers by selective elimination of lymphoid cells in donor marrow. Transplant. Proc. *8,* 537 (1976)
23. National Council on Radiation Protection and Measurements: Basic radiation criteria. NCRP Report *39,* NRCP Publications, Washington D.C. (1971)
24. Pegg, D. E.: Bone marrow transplantation. London: Lloyd-Luke Ltd. 1966
25. Nowell, P. C., Cole, L. J., Habermeyer, J. G., Roan, P. L.: Growth and continued function of rat marrow cells in X-irradiated mice. Cancer Res. *16,* 258–261 (1956)
26. Rieder, I., Kolb, H. J., Schaffer, E., Kolb, H., Grosse-Wilde, H., Scholz, S., Thierfelder, S.: Leukocyte transfusions for the modification of host-versus-graft reactions in dogs. In: Baum, S. J., Ledney, G. D. (Eds.): Experimental Hematology Today 1978. New York: Springer 1978
27. Santos, G. W., Owens, A. H., jr.: Syngeneic and allogeneic marrow transplants in the cyclophosphamide pretreated rat. Adv. Transplant. *28,* 431 (1968)
28. Sharkis, S. J., Santos, G. W.: Bone marrow transplantation in a BN rat model of acute myelogenous leukemia (AML). Leukemia Research *1,* 251 (1977)
29. Sonneveld, P., Bekkum, D. W., van: The effect of whole body irradiation on skeletal growth in rhesus monkeys. Radiology *130,* 789 (1979)
30. Sonneveld, P., Peperkamp, E., Bekkum, D. W., van: Incidence of cataracts in rhesus monkeys treated with whole body irradiation. Radiology *133,* 227 (1979)
31. Storb, R., Epstein, R. B., Bryant, J., Ragde, H., Thomas, E. D.: Marrow grafts by combined marrow and leukocyte infusions in unrelated dogs selected by histocompatibility typing. Transplantation *6,* 587 (1968)
32. Storb, R., Weiden, P. L., Graham, T. C., Lerner, K. G., Nelson, N., Thomas, E. D.: Hemopoietic

grafts between DLA-identical canine littermates following dimethyl-myleran. Transplantation *24*, 349 (1977)
33. Thilsted, J. P., Shifrine, M., Wilson, F. D.: Induction of Graft-versus-Host Disease in immunocompetent dogs with lymphocytes from BCG-immunized donors – 1977. Annual Report Radiobiology Laboratory, p. 111. Davis: University of California 1977
34. Thomas, E. D., Buckner, C. D., Benaji, M., Clift, R. A., Fefer, A., Flournoy, N., Goodell, B. W., Hickman, R. O., Lerner, K. G., Neiman, P. E., Sale, G. E., Sanders, J. E., Singer, J., Stevens, M., Storb, R., Weiden, P. L.: One hundred patients with acute leukemia treated by chemotherapy, total body irradiation and allogeneic marrow transplantation. Blood *49*, 511 (1977)
35. Torok-Storb, B. J., Storb, R., Graham, T. C., Prentice, R. L., Weiden, P. L., Adamson, J. W.: Erythropoiesis in vitro: Effect of normal versus transfusion-sensitized mononuclear cells. Blood *52*, 706 (1978)
36. Vos, O., Davids, J. A. G., Weyzen, W. W. H., Bekkum, D. W., van: Evidence for the cellular hypothesis in radiation protection by bone marrow cells. Acta Physiol. Pharmacol. Neerl. *4*, 482 (1956)
37. Vriesendorp, H. M., Rothengatter, C., Bos, E., Westbroek, D. L., Rood, J. J., van: The production and evaluation of dog allolymphocytotoxins for donor selection in transplantation experiments. Transplantation *11*, 440 (1971)
38. Vriesendorp, H. M., Bijnen, A. B., Zurcher, C., Bekkum, D. W., van: Donor selection and bone marrow transplantation in dogs. In: Kissmeyer-Nielsen, F. (Ed.): Histocompatibility Testing 1975, pp. 963. Copenhagen: Munksgaard 1975
39. Vriesendorp, H. J., Zurcher, C., Bull, R. W., Los, W. K. F., Meera-Khan, P., Tweel, J. G., v.d., Zweibaum, A., Bekkum, D. W., van: Take and graft vs. host reactions of allogeneic bone marrow in tissuetyped dogs. Transplant. Proc. *7*, Suppl. 1, 849 (1975)
40. Vriesendorp, H. M., Löwenberg, B., Visser, T. P., Knaan, S., Bekkum, D. W., van: Influence of genetic resistance and silica particles on survival after bone marrow transplantation. Transplant. Proc. *8*, 483 (1976)
41. Vriesendorp, H. M., Bijnen, A. B., Kessel, A. C. M., van, Obertop, H., Westbroek, D. L.: Minor histocompatibility systems in dogs. In: Baum, S. L., Ledney, G. D. (Eds.): Experimental Hematology Today 1978, p. 109. Berlin. Heidelberg. New York: Springer 1978
42. Weiden, P. L., Storb, R., Graham, T. C., Sale, G. E., Thomas, E. D.: Resistance to DLA-nonidentical marrow grafts in lethally irradiated dogs. Transplant. Proc. *9*, 285 (1977)
43. Whitmore, G. F., Till, J. E.: Quantitation of cellular radiobiological responses. Ann. Rev. Nuclear Sci. *14*, 347 (1964)

Discussion

Fliedner: Don't you think that it is more appropriate these days to express the bone marrow cell dose in numbers of progenitor cells? Furthermore, don't you think that it would be better to measure bone marrow cellularity instead of a 50 per cent survival thereby excluding infections and other causes contributing to death in these animals? We have stressed already 1965, that there is a basic difference in kinetics of bone marrow restoration between man and all animal species. Using peripheral blood stem cell we have not found a difference in numbers required to restore autologous or allogeneic recipients. Finally, we have found the D_0 for CFU-C in vitro to be possibly below 50 rads.

Vriesendorp: At this time, I would prefer to use bone marrow cell numbers instead of CFU-C, since the tests of CFU-C and the results vary considerably between different laboratories. The use of bone marrow cellularity in preference to survival is illogical, since there are lot of irrelevant cells in bone marrow and its only the hemopoietic stem cell in which we are interested. Looking at the Seattle data of leukocyte and platelet recovery I could not find basic differences in the kinetics of restoration as compared to animal data.

Storb: Looking at the overall leukemic patients in Seattle there is no correlation of cell dose and GVHD. Furthermore, in the aplastic anemia patients which is a cleaner model there is absolutely no correlation of the cell dose with survival nor GVHD in patients with engraftment. Finally, GVHD is

a multifactorial event which requires in my opinion a more sophisticated statistical analysis than a Fishers exact test.

Vriesendorp: The aplastic anemia patients are uncomparable because of Cyclophosphamide conditioning. I dont think that the correlation of cell dose to GVHD has been proven in man, but taking animal data into account, it may be worthwile to study lower cell doses in man.

8 Allogeneic Bone Marrow Transplantation for the Treatment of Leukemia, Aplastic Anemia and Immunodeficiency

Recent Results in Marrow Transplantation for the Treatment of Aplastic Anemia and Acute Leukemia in Seattle*

R. Storb
for the Seattle Marrow Transplant Team

The major impediment to uniformly successful application of marrow transplantation to man is the genetically determined donor-host histoincompatibility. This is not a problem when donor and recipient are gentically identical twins (a syngeneic graft) – the infused marrow is readily accepted. Simple infusion of the twin marrow, therefore, is effective treatment for aplastic anemia. However, when donor and recipient are *not* monozygous twins (an allogeneic graft), some degree of histoincompatibility exists and constitutes a bidirectional immunologic barrier.

Based on studies in rodents and dogs, allogeneic donors have been almost exclusively family members compatible with the recipient for the antigens of the major human histocompatibility complex, HLA-A and -B and mutually nonstimulatory in mixed leukocyte culture (MLC). Studies in dogs [12] predicted that some patients would develop GVHD presumably due to genetic determinants outside of HLA which are not detected by present histocompatibility typing techniques. Successful transplants from unrelated donors for the treatment of aplastic anemia or leukemia have so far not been reported.

The immune defense system of the patient must be suppressed for the allogeneic marrow graft to be accepted. Presently, two methods are used in clinical practice: intravenous injection of cyclophosphamide (CY), and total body irradiation (TBI). The dose of CY is high, 50 mg/kg body weight on each of 4 successive days. TBI is given either alone or in conjunction with chemotherapeutic agents and administered at a dose of 1000 rad midline tissue at a rate of 4 to 8 rads/minute by opposing ^{60}Co irradiation sources which are not available in many clinical centers. In the case of the patient with leukemia, the conditioning regimens also serve to eradicate the leukemic cells.

The immediate post-grafting period is characterized by a lack of blood cell production and, consequently, by the danger of infection and hemorrhage. Frequent platelet transfusions are necessary to maintain the level of platelets above 20,000/mm^3. Prophylactic granulocyte transfusions and laminar air flow isolation have decreased the incidence of post-transplant infections although their influence on overall survival is less clear.

A major problem has been that of GVHD. Based on animal experiments [11], the immunosuppressive agent methotrexate (MTX) is administered intermittently for approximately 100 days post-grafting. After discontinuation of MTX many

* This investigation was supported by grant Nos. CA 18029, CA 17117, CA 15704 and CA 18221, awarded by the National Cancer Institute, DHEW

patients show persistence of immunologic tolerance between the donor and the host cells. The nature of this immunologic tolerance has as yet not been fully elucidated. In a number of patients, however, life-threatening GVHD, acute and chronic, has been seen despite MTX. Based on canine studies, rabbit, goat or horse antihuman antithymocyte globulin (ATG) has been used to treat established human GVHD with some success. Prednisone also appears to be of value. The development of fatal interstitial pneumonias, either of unknown etiology or associated with cytomegalovirus and Pneumocystis carinii infections has remained a major problem. The current report summarizes the results obtained in Seattle.

A. Aplastic Anemia

Marrow transplantation for the patient with severe aplastic anemia is an effective form of treatment for the patient who has a suitable marrow donor. Two of the first 4 allogeneic transplant recipients [20] in the Seattle series are now well more than 8 years post-grafting [14]. A prospective cooperative study has shown a significantly better survival for patients treated by transplantation than for those who were not transplanted because of the lack of a suitable donor [1].

Severe aplastic anemia was defined as a hypoplastic marrow with at least 2 of the following characteristics: Granulocyte count less than 500/mm^3; platelet count less than 20,000/mm^3; and reticulocyte count less than 1 per cent in the presence of anemia. Patients with these findings seem to have a poor prognosis with conventional therapy although recovery of an occasional patient with severe aplastic anemia has been reported. The currently used prognostic factors are not yet sufficient to separate with certainty those patients destined for spontaneous recovery from those with a fatal outcome [1, 6, 7, 26].

Clinical Results of Marrow Transplantation

One-hundred and fifty-seven consecutive patients with severe aplastic anemia have been treated in Seattle by marrow transplants from HLA identical family members after CY and/or TBI [13–16, 17, 20]. Seventy-three of the patients

Table 1. Results in patients with aplastic anemia conditioned with Cy

Group of Patients	Number of Pts. studied	% of patients	
		With graft rejection	Surviving
Before October 1975	63	32	46
After October 1975 "non-sensitized" (marrow only)	47	17	73
"Sensitized" (marrow plus viable donor buffy-coat)	27	11	74

were grafted before October 1975, and 43% are surviving. Of these 73, 63 were conditioned by CY (Table 1) and 10 by TBI. One problem associated with high mortality has been marrow graft rejection seen in 21 patients (29%) which was fatal in 19. A multi-factorial regression analysis of data on the 73 patients showed that 2 factors strongly predicted graft rejection [17]. One was positive in vitro tests of cell-mediated immunity [8, 24] indicating sensitization of marrow recipient against marrow donor, presumably reflecting transfusion-induced sensitization to non-HLA antigens. Eighty per cent of patients with positive in vitro test results rejected and only 25% survived. The other factor was a low number of marrow cells (less than 3×10^8 cells/kg) used for transplantation.

The analysis suggested that patients with aplastic anemia who have an HLA identical sibling should be transplanted early, before transfusions are administered and sensitization to the marrow donor can develop. Accordingly, 30 non-transfused patients have been transplanted in Seattle. One patient died of infection. Three died with complications associated with acute or chronic GVHD. Twenty-six are alive with good hematologic function between 3 and 77 (median 13) months after grafting [16 and Storb et al. Ann. Int. Med., in press].

After October 1975 the marrow transplant regimens were revised. "Non-sensitized" patients with little likelihood of graft rejection (negative in vitro tests) were conditioned with Cy alone while "sensitized" patients with a high likelihood of graft rejection (positive in vitro tests) were conditioned with a more aggressive regimen consisting of either a combination of procarbazine, antithymocyte globulin and TBI or a combination of Cy and TBI. Eleven "sensitized" patients were so treated with only one rejecting. However, survival was poor because of complicating graft-vs-host disease (GVHD) and interstitial pneumonia. The use of the TBI regimen was, therefore, suspended.

We then investigated the usefulness of putative hemopoietic stem cells derived from the peripheral blood. Previous work in mice, guinea pigs, dogs and baboons has shown that pluripotent hemopoietic stem cells circulate in the blood [2, 5, 9, 10, 18]. Studies in man documented the presence of committed hemopoietic stem cells in the circulating blood [3, 4]. Thus, the use of viable mononuclear cells from the peripheral blood of the marrow donor in addition to the marrow inoculum, appeared to be a reasonable approach to increase the number of stem cells infused and decrease the rejection rate.

Table 1 summarizes the results in patients conditioned with CY and transplanted before and after October 1975. The rejection rate in patients grafted before October 1975 was 32% and survival 46%. After October 1975, rejection in "non-sensitized" patients was 17% and survival 73%. Twenty-seven "sensitized" patients were given marrow plus approximately 2×10^8 viable mononuclear peripheral blood leukocytes/kg collected from the marrow donor over a 3–5 day period. Rejection was 11% and survival 74%.

An important question was whether the GVHD incidence was increased after buffy coat infusion. To this purpose, results in all patients with sustained engraftment transplanted since 1975 after CY were considered. Patients not given buffy coat were compared to those given donor buffy coat. No difference in the incidence of GVHD between the two groups was detected. Eighty-four per cent of the patients with sustained engraftment are surviving regardless of

whether they received buffy coat or not. Thus, buffy coat has decreased the rejection rate and increased survival without a concomittant increase in GVHD.

In conclusion, survival after marrow transplantation for aplastic anemia has increased to 73%. One cause of success has been the recognition and apparent effective reduction of marrow rejection. Another reason is a decrease in the mortality from GVHD for which no immediate explanation is identifiable. These results suggest that aplastic anemia regardless of the various etiologic factors implicated, is usually related to a failure of the hemopoietic stem cells that can be corrected by infusion of marrow from a normal donor. Clearly, major problems in transplantation remain to be solved such as avoiding or abrogating sensitization by transfusions and thereby further decreasing the incidence of graft rejection, prevention and control of acute and chronic GVHD and forestallment or management of infection in the immunosuppressed patient. Even so, the survival and hematologic reconstitution of currently 73% of the patients with severe aplastic anemia indicate that marrow transplantation is more effective than conventional management of this disease.

One important step to improve the overall clinical results is for the physician to be aware of the possibility of marrow transplantation when a patient with aplastic anemia is first seen. HLA typing of patient and family should be done immediately. If no HLA identical family member is available, marrow transplantation should not be attempted at present and conventional treatment can be instituted. If, however, an HLA identical family member is available, early transplantation should be considered. Transfusions of blood products should not be given unless indicated by urgent medical necessity. All transfusions from family members should be avoided. If transfusions are necessary, washed, buffy-coat poor red blood cells and platelets from single donors should be used.

B. Acute Leukemia

Marrow transplantation for acute leukemia involves the same general transplantation biology problems that are encountered in aplastic anemia, however, graft rejection is rare. The additional major problem is the eradication of leukemia. Despite these problems, an increasing number of long-term survivors is being seen. Until recently, marrow transplantation for treatment of leukemia was carried out only after failure of all other therapy. Consequently, most patients were in advanced relapse with a heavy burden of leukemic cells. Despite these obstacles the longest disease free survivor is now 8½ years after transplantation, and in the United States there are 15 leukemia free patients who are now more than 4 years post-transplantation. None of the patient had any chemotherapy after transplantation.

Clinical Results

The first 10 patients were conditioned for grafting by 1000 rads of TBI and the subsequent 100 patients were prepared with CY (60 mg/kg on each of two days) followed by TBI [21]. An actuarial survival curve of these patients by the method

of Kaplan and Meier shows three periods of interest [22]. The first period concerns the first 120 days after transplantation with a rapid loss of patients related to advanced illness, GVHD and associated infections and, to a lesser extent, recurrent leukemia. The second period extending from approximately 120 days to 2 years post-grafting shows a much slower rate of loss of patients, primarily due to recurrent leukemia. The third period, which now extends from 4 to 8½ years, is almost flat with a negligible loss of patients and no recurrent leukemia. This flat portion of the curve, which corresponds to about 15% of the patients, constitutes an operational definition of cure of these patients with otherwise refractory leukemia.

In an attempt at reducing the leukemic relapse after marrow transplantation, a number of patients received further chemotherapy, principally rubidomycin, cytosine arabinoside and bis-chloroethyl-nitrosourea. Although the number of patients is too small for critical analysis, added chemotherapy was toxic and increased the time of maximal pancytopenia, and instances of recurrent leukemia continued to be observed.

Current efforts in Seattle for reducing the incidence of recurrent leukemia and increasing the number of long-term survivors are twofold. One involves the use of a much higher dose of TBI for the patient in relapse, by means of fractionating the irradiation. The total irradiation doses given at present are on the order of 1400–1500 rad. These doses are quite well tolerated. However, patient follow-up is too short to draw definite conclusions as to the validity of this approach. It could be that these efforts are misdirected, since, in an exponential killing process, it is difficult to kill the last leukemic cell. The apparent cures may be due to eradication of the last leukemic cell by an immunologic mechanism involving minor transplantation antigens and/or leukemia associated antigens. Such a possibility is supported by recent observations of a graft-versus-leukemia effect in man [25]. These studies showed that allogeneic marrow recipients with moderate to severe GVHD were significantly less likely to develop leukemic relapse than were allogeneic marrow recipients with no or mild GVHD or syngeneic marrow recipients. This apparent antileukemic effect of GVHD was stronger among patients with ALL than among patients with ANL, and present mainly among patients transplanted in leukemic relapse. The antileukemic effect of GVHD was most evident during the first 130 days post-transplantation. Survival of patients with moderate to severe GVHD was comparable to that of patients with no or minimal GVHD, since their lesser probability of recurrent leukemia was offset by a greater likelihood of other causes of death, primarily interstitial pneumonia.

The second approach involves marrow transplantation before the patient reaches the endstages of the disease. The advantages of this approach include
1. Treatment before the leukemic cell population becomes refractory to therapy,
2. treatment when the body burden of leukemic cells is minimal, and
3. treatment while the patient is in excellent clinical condition and, therefore, better able to tolerate the transplantation regimen. In January of 1976 a Seattle protocol for early transplantation was approved by the Human Subjects Review Committee of the University of Washington. This protocol involves CY plus TBI and marrow transplantation for patients with AML in the first or subsequent

Table 2. Allogeneic Marrow Transplants for Leukemia in Remission

Disease	No. of patients studied	No. of pts with relapse after transplantation	No. living in remission	Survival range (months)
ALL	22	10	11	12–31
AML	23	3	13	12–36
Total	45	13	24	12–36

remission and for patients with ALL in the second or subsequent remission. So far, 23 patients with AML and 22 with ALL have been grafted [23]. The results are summarized in Table 2. The leukemic recurrence rate is significantly lower and the survival significantly better than that of 50 patients transplanted concurrently in relapse. The improved survival of the patients transplanted in remission indicates that the risks and complications of marrow grafting are offset by the probability of a long-term remission. For the patient with an HLA identical sibling, marrow transplantation should be undertaken before the stage of terminal relapse is reached.

References

1. Camitta, B. M., Thomas, E. D., Nathan, D. G., Santos, G., Gordon-Smith, E. C., Gale, R. P., Rappeport, J. M., Storb, R.: Severe aplastic anemia: A prospective study of the effect of early marrow transplantation on acute mortality. Blood *48*, 63–70 (1976)
2. Cavins, J. A., Scheer, S. C., Thomas, E. D., Ferrebee, J. W.: The recovery of lethally irradiated dogs given infusions of autologous leukocytes preserved at −80 C. Blood *23*, 38–43 (1964)
3. Chervenick, P. A., Boggs, D. R.: In vitro growth of granulocytic and mononuclear cell colonies from blood of normal individuals. Blood *37*, 131–135 (1971)
4. Golde, D. W., Cline, M. J.: Identification of the colony-stimulating cell in human peripheral blood. J. Clin. Invest. *51*, 2981–2983 (1972)
5. Goodman, J. W., Hodgson, G. S.: Evidence for stem cells in the peripheral blood of mice. Blood *19*, 702–714 (1962)
6. Lohrmann, H.-P., Kern, P., Niethammer, D., Heimpel, H.: Identification of high-risk patients with aplastic anaemia in selection for allogeneic bone-marrow transplantation. Lancet *2*, 647–650 (1976)
7. Lynch, R. E., Williams, D. M., Reading, J. C., Cartwright, G. E.: The prognosis in aplastic anemia. Blood *45*, 517–528 (1975)
8. Mickelson, E. M., Fefer, A., Storb, R., Thomas, E. D.: Correlation of the relative response index with marrow graft rejection in patients with aplastic anemia. Transplantation *22*, 294–300 (1976)
9. Storb, R., Epstein, R. B., Ragde, H., Bryant, J., Thomas, E. D.: Marrow engraftment by allogeneic leukocytes in lethally irradiated dogs. Blood *30*, 805–811 (1967)
10. Storb, R., Epstein, R. B., Bryant, J., Ragde, H., Thomas, E. D.: Marrow grafts by combined marrow and leukocyte infusions in unrelated dogs selected by histocompatibility typing. Transplantation *6*, 587–593, 1968
11. Storb, R., Epstein, R. B., Graham, T. C., Thomas, E. D.: Methotrexate regimen for control of graft-versus-host disease in dogs with allogeneic marrow grafts. Transplantation *9*, 240–246 (1970)
12. Storb, R., Rudolph, R. H., Thomas, E. D.: Marrow grafts between canine siblings matched by serotyping and mixed leukocyte culture. J. Clin. Invest. *50*, 1272–1275 (1971)

13. Storb, R., Thomas, E. D., Buckner, C. D., Clift, R. A., Johnson, F. L., Fefer, A., Glucksberg, H., Giblett, E. R., Lerner, K. G., Neiman, P.: Allogeneic marrow grafting for treatment of aplastic anemia. Blood *43,* 157–180 (1974)
14. Storb, R., Thomas, E. D., Buckner, C. D., Clift, R. A., Fefer, A., Fernando, L. P., Giblett, E. R., Johnson, F. L., Neiman, P. E.: Allogeneic marrow grafting for treatment of aplastic anemia: A follow-up on long-term survivors. Blood *48,* 485–490 (1976)
15. Storb, R., Thomas, E. D., Weiden, P. L., Buckner, C. D., Clift, R. A., Fefer, A., Fernando, L. P., Giblett, E. R., Goodell, B. W., Johnson, F. L., Lerner, K. G., Neiman, P. E., Sanders, J. E.: Aplastic anemia treated by allogeneic bone marrow transplantation: A report on 49 new cases from Seattle. Blood *48,* 817–841 (1976)
16. Storb, R., Thomas, E. D., Buckner, D., Fefer, A., Goodell, B., Neiman, P., Sanders, J., Singer, J., Weiden, P.: Marrow transplantation in untransfused patients with severe aplastic anemia (AA). Blood *50* (Suppl. 1), 316 (1977)
17. Storb, R., Prentice, R. L., Thomas, E. D.: Marrow transplantation for treatment of aplastic anemia. An analysis of factors associated with graft rejection. N. Engl. J. Med. *292,* 61–66 (1977)
18. Storb, R., Graham, T. C., Epstein, R. B., Sale, G. E., Thomas, E. D.: Demonstration of hemopoietic stem cells in the peripheral blood of baboons by cross circulation. Blood *50,* 537–542 (1977)
19. Storb, R., Thomas, E. D. for the Seattle Marrow Transplant Team: Marrow transplantation for treatment of aplastic anemia. In: Thomas, E. D. (Ed.): Clinics in Haematology, pp. 597–609. London: W. B. Saunders Comp. Ltd. 1978
20. Thomas, E. D., Buckner, C. D., Storb, R., Neiman, P. E., Fefer, A., Clift, R. A., Slichter, S. J., Funk, D. D., Bryant, J. I., Lerner, K. G.: Aplastic anaemia treated by marrow transplantation. Lancet *1,* 284–289 (1972)
21. Thomas, E. D., Buckner, C. D., Banaji, M., Clift, R. A., Fefer, A., Flournoy, N., Goodell, B. W., Hickman, R. O., Lerner, K. G., Neiman, P. E., Sale, G. E., Sanders, J. E., Singer, J., Stevens, M., Storb, R., Weiden, P. L.: One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. Blood *49,* 511–533 (1977)
22. Thomas, E. D., Flournoy, N., Buckner, C. D., Clift, R. A., Fefer, A., Neiman, P. E., Storb, R.: Cure of leukemia by marrow transplantation. Leukemia Research *1,* 67–70 (1977)
23. Thomas, E. D., Sanders, J. E., Flournoy, N., Johnson, F. L., Buckner, C. D., Clift, R. A., Fefer, A., Goodell, B. W., Storb, R., Weiden, P. L.: Marrow transplantation for patients with acute lymphoblastic leukemia in remission. Blood *54,* 468–476 (1979)
24. Warren, R. P., Storb, R., Weiden, P. L., Mickelson, E. M., Thomas, E. D.: Direct and antibody-dependent cell-mediated cytotoxicity against HLA identical sibling lymphocytes. Correlation with marrow graft rejection. Brief Communication. Transplantation *22,* 631–635 (1976)
25. Weiden, P. L., Doney, K., Storb, R., Thomas, E. D. for the Seattle Marrow Transplant Group: Antihuman thymocyte globulin (ATG) for prophylaxis of graft-versus-host disease: A randomized trial in patients with leukemia treated with HLA identical sibling marrow grafts. Transplantation *27,* 227–230 (1979)
26. Williams, D. M., Lynch, R. E., Cartwright, G. E.: Drug-induced aplastic anemia. Semin. Hematol. *10,* 195–223 (1973)

Discussion

Kolb: Did you see reversal among your Cyclophosphamide treated patients and could you relate reversion to the addition of buffy coat? We have not seen reversals in our dogs given buffy coat in addition.

Storb: There have been 4 reversals among 150 patients, in three of them reversion is complete, one patient has reversed with regard to his bone marrow, but is still a mixed chimera in his blood. Among 27 patients given buffy coat we have not seen a reversion yet. One of the reversals was a nontransfused patient.

Heimpel: Which cells in buffy coat influence the rejection? Immunocompetent cells, stem cells or all together?
Storb: My feeling is that it is a lymphoid cell.
Kaizer: Do you have data on identical twin transplants in remission as compared to those in relapse?
Storb: We have done 15 patients in remission and the results are similar to those presented here.
Kay: The data for chronic GVHD in patients over the age of 30 are depressing. Did you modify your protocol for patients over the age of 30?
Storb: We have included all patients, those with slight chronic GVHD, those with transient GVHD as well as those with severe GVHD. The overall figure of 35 per cent may be misleading. A third of the patients treated with combination chemotherapy required no longer therapy for chronic GVHD.
Kersey: There has been a double incidence rate of chronic GVHD in patients given buffy coat. Is this entirely related to the buffy coat or are there other factors like age and so on?
Storb: No, in the old days we lost 30 per cent of our patients with acute GVHD, now we loose only 5 per cent. With improvement of preventing acute GVHD we see more chronic GVHD. The figures of chronic GVHD in nontransfused patients given marrow only are not different from those of patients given buffy coat in addition.
Halle-Pannenko: Is GVHD increased in the sensitized recipients as compared to non-sensitized recipients?
Storb: No.
Halle-Pannenko: In mice you get more severe GVHD by adding lymphocytes. How do you explain that you get not more GVHD by adding buffy coat?
Storb: Dog experiments showed that we did not increase the incidence of GVHD by adding buffy coat, but GVHD occurred earlier. This effect was blunted by Methotrexate.
Halle-Pannenko: Have you data on HLA-identical unrelated transplants?
Storb: In humans? No.
Vriesendorp: You changed your rejection rate from over 30 per cent to 11 per cent. How much is due to higher bone marrow doses and how much addition of buffy coat?
Storb: In the past the overage bone marrow dose was 3.6×10^8 per kilogram, now it is $3{,}85 \times 10^8$ per kg. So we have not changed the marrow dose. Furthermore, comparing nonsensitized patients given marrow alone with sensitized patients given buffy coat in addition we saw a lower rejection rate in the latter. So, I think it is the buffy coat.
Dicke: What is the median age in your patients transplanted in remission? Is there an age limit for remission transplants?
Storb: It is about 21 for patients with ANL and about 17 years for patients with ALL, we have done only four or five patients over the age of 40.
Dicke: Our 3 year survival in conventionally treated patients of this favorable age group is about 28 per cent.

Marrow Transplantation in Acute Leukemia Following Busulfan and Cyclophosphamide*

P. J. Tutschka, G. W. Santos, and G. J. Elfenbein

A. Introduction

In 1968 we introduced allogeneic bone marrow transplantation after conditioning with drugs alone (Santos et al., 1979). Cyclophosphamide was used solely, in very high doses (60 mg/kg, later 50 mg/kg daily for four days), to prepare leukemic patients to accept marrow grafts from donors matched at the major histocompatibility complex, a protocol which ultimately served as a major conditioning regimen for grafting patients with severe aplastic anemia (Santos et al., 1974; Storb et al., 1978).

The immunologic background and the rationale for this approach had been derived from animal models, especially a rat model of allogeneic bone marrow transplantation that appeared to resemble quite well the clinical situation (Santos, Owens 1968). This animal model was used over the subsequent years to further define the principles governing allogeneic marrow transplantation and to improve the transplantation protocols for clinical use (Santos, Owens, 1966; Santos, Tutschka, 1974a; Santos, Tutschka, 1974b; Tutschka, Santos, 1975a; Tutschka, Santos, 1975b; Tutschka, Santos, 1977). This report describes some of the pertinent findings in our rat model leading to major modifications of our initial protocol. Furthermore, it will summarize results of our human marrow transplantation studies using conditioning with drug alone to prepare leukemic recipients for marrow grafting.

B. Animal Studies

Groups of Lewis rats (Ag-B1) were given graded single doses of cyclophosphamide (Cy) (50, 100, 150 and 200 mg/kg) followed by the infusion of 60×10^6 viable nucleated marrow cells from AgB histoincompatible ACI (Ag-B4) donors. The animals were observed daily and their cumulative mortality and incidence of clinically and histopathologically demonstrable graft-versus-host disease (GVHD) were recorded. Furthermore, the evidence for engraftment, completeness of take and kinetics of marrow graft establishment were evaluated by typing with antisera specific for ACI immunoglobulin allotype (Humphrey, Santos, 1971) and by typing the peripheral lymphocytes of the graft recipients with strain specific cytotoxic isoantisera (Tutschka, Santos, 1977).

---

* Supported by ACS grant No. CH-113 and NIH-NCI grant No. CA-15396

Table 1. Transplantation of ACI marrow in Lewis rats conditioned with graded doses of CY[a]

Dose of Cy	Days after transplantation								
	14			28			70		
mg/kg	Mort.[b]	Chim.[c]	GVHD[d]	Mort.	Chim.	GVHD	Mort.	Chim.	GVHD
Cy 50	0/20	0/20	0/20	0 0	0/20	0/20	0 0	0/20	0 0
Cy 100	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20
Cy 150	0/20	0/20	0/20	2/20	5/18	2/18	2/20	2/18	0/18
Cy 200	1/20	11/19	8/19	12/20	6/8	4/8	12/20	7/8	0/8

[a] Lewis rats were given graded single doses of Cy 24 hr before the infusion of 64×10^6 nucleated marrow cells

[b] Number of animals dead over total (Mort, mortality)

[c] Number of survivors with ACI immunoglobulin over total surviving (Chim, chimerism)

[d] Number of survivors with clinical GVHD over total surviving

A clear relation between the dose of cyclophosphamide, the evidence of engraftment and the incidence of GVHD could be demonstrated (Table 1). No engraftment was seen with the lowest dose of Cy, 50 mg/kg, and only transient engraftment with 100 and 150 mg/kg. Permanent takes were seen at a dose of 200 mg/kg with a mixture of donor and recipient cells early on, gradual increase in the fraction of donor cells over the course of time and finally complete replacement of recipient type cells by donor type cells (Fig. 1).

Ford et al. (1966) had shown the preferential engraftment of infused marrow in the irradiated but not shielded partial body irradiated recipients, a phenomenon that was confirmed in similar studies by Kolb, et al. (Kolb et al., 1972) and

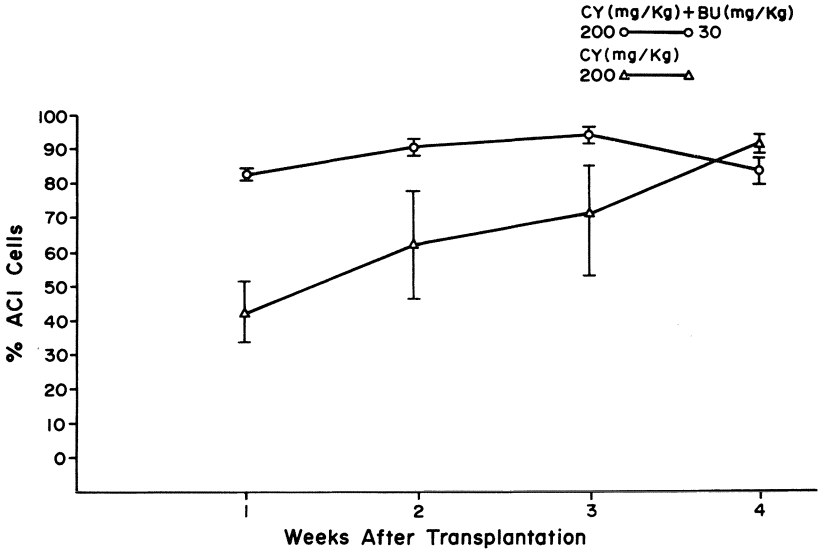


Fig. 1. Kinetics of engraftment after conditioning with Cy and Cy + BU. Shown are the percent ACI lymphocytes in the peripheral blood of Lewis recipients at various weeks after transplantation

Slavin (1979). Based on those results we suggested that "space" making was an important prerequisite for an optimal conditioning regimen to assure prompt and complete marrow allograft acceptance (Tutschka, Santos, 1973). To prove this concept busulfan was added to the cyclosphosphamide regimen, a drug that is highly myelotoxic and at most only minimally immunosuppressive in the rat (Santos, Tutschka, 1974a; Santos, Tutschka, 1974b; Tutschka, Santos, 1975a) and presumably acts toxic exclusively to the myeloid stem cell (Dexter, Spooncer, 1979).

Lewis rats were given graded doses of cyclophosphamide (50, 100, 150, 200 mg/kg) together with a highly myeloablative dose of 30 mg/kg busulfan 24 hours prior to infusion of ACI marrow cells. Most animals given 50 mg/kg died from marrow aplasia, presumably because of insufficient immunosuppression to allow a take. Evidence of engraftment was seen with as little as 100 mg/kg Cy, and complete, persistent chimerism was seen with 150 mg/kg Cy, a dose that would not allow permanent engraftment when given by itself. The markedly improved quality of engraftment after addition of BU is seen in Figure 1, which shows the kinetics of engraftment after conditioning with Cy and Cy + BU. Compared to the gradual establishment of the graft after conditioning with Cy only addition of BU leads to prompt and complete grafts very early after marrow transplantation.

Table 2. Transplantation of ACI marrow in Lewis rats conditioned with graded doses of CY and 30/mg/kg BU[a]

Dose of Cy	Days after transplantation								
	14			28			70		
mg/kg	Mort.[b]	Chim.[c]	GVHD[d]	Mort.	Chim.	GVHD	Mort.	Chim.	GVHD
Cy 50+BU	20/20	–	–	–	–	–	–	–	–
Cy 100+BU	2/20	14/18	0/18	16/20	4/4	4/4	20/20	–	–
Cy 150+BU	0/20	16/20	0/20	10/20	10/10	8/10	20/20	–	–
Cy 200+BU	6/20	14/14	10/14	16/20	4/4	4/4	20/20	–	–

[a] Lewis rats were given 30 mg/kg of BU per kg plus graded single doses of Cy 24 hr before the infusion of 64×10^6 nucleated marrow cells

[b] Number of animals dead over total (Mort, mortality)

[c] Number of survivors with ACI immunoglobulin over total surviving (Chim, chimerism)

[d] Number of survivors with clinical GVHD over total surviving

C. Human Studies

While those animal studies were carried out we attempted allogeneic marrow transplantation in a series of patients suffering from leukemia, the only conditioning regimen administered being cyclophosphamide. Twentyfour patients, seven with acute lymphocytic and 17 with acute non-lymphocytic leukemia, received 200 mg/kg of Cy given in four daily doses of 50 mg/kg followed by infusion of genotypically HLA identical sibling marrow 36–48 hours after completion of the administration of Cy.

The initial antileukemic efficacy of this regimen as defined by disappearance of leukemic cells in bone marrow and peripheral blood one week after drug administration, was excellent and 23/24 patients cleared the tumor initially. Complete engraftment as defined by appearance of donor cells in marrow and peripheral blood or signs of graft-versus-host reaction on biopsy and recovery of peripheral white cell and platelet count was seen in 21 of the 24 patients. Fatal graft-versus-host disease occurred in four and fatal interstitial pneumonia in only three patients. A total of 12 patients, however, had recurrence of the leukemia, and all of the seven patients who survived more than 150 days showed ultimately fatal recurrence of the leukemia.

Since cyclophosphamide as the sole conditioning agent had an unacceptably high rate of leukemia recurrence we added another agent with antileukemic efficacy to the preparative regimen. Based on our animal studies we chose busulfan hoping not only to reduce the incidence of leukemia recurrence but also to improve quality and kinetics of engraftment. Fifteen patients with acute non-lymphocytic leukemia received a combination regimen of 8–20 mg/kg BU given over 4–8 days followed by 200 mg/kg Cy given in four daily doses of 50 mg/kg. The initial antileukemic effect was excellent with 14/15 patients showing clearing of the tumor. Three patients died too early to evaluate, the remaining 12 showed complete engraftment. Fatal GVHD was seen in three and fatal interstitial pneumonia in five patients. The cumulative recurrence rate of leukemia was only 2/15, and to date (4/1/79) two patients have survived free of leukemia 309+ and 641+ days after marrow grafting.

Table 3. Marrow transplantation in leukemic patients conditioned with CY or CY+BU

Regimen	No. of patients	Anti-leukemic effect	Engraftment	Fatal GVHD	Fatal interstitial pneumonitis	Observed leukemia rates total	Observed leukemia rates >150 days	Survival percent (1/4/79)
Cy	24	23/24	21/24	4/21	3/21	12/24	7/7	0%
Cy+BU	15	14/15	12/12	3/12	5/12	2/15	0/2	13%

Table 3 compares patients conditioned with Cy and Cy+BU.

As predicted from the above described animal model adding BU to the Cy regimen appeared to accelerate the establishment of the graft. A gradual establishment of complete chimerism was found in patients with Cy after examining the percent of donor karyotypes in blood and bone marrow of the patients post transplant (Fig. 2). Addition of BU to Cy rapidly established a complete graft, a finding that correlated with the kinetics of white cell recovery after transplantation: patients conditioned with Cy alone required a median of 26 days to reach a white blood cell count of $1000/mm^3$, whereas patients conditioned with BU+Cy revocered in only 16 days (Fig. 3).

It is unclear how busulfan brings about these remarkable changes. We have termed its effect "space making", an operational term that could include myeloablative properties creating actual physical room (Tutschka, Santos, 1973), putative helper effects for the stromal microenvironment thus favoring more

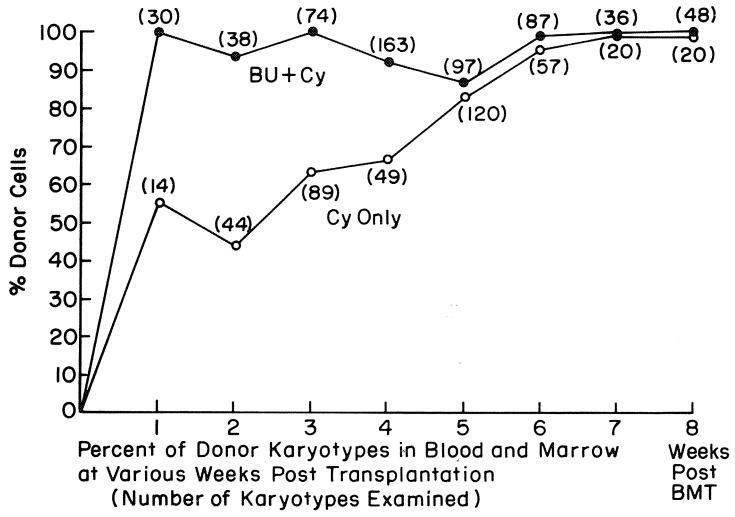


Fig. 2. Kinetics of engraftment after conditioning leukemic patients with Cy and Cy + BU. Shown are the percent donor cells in blood and marrow of patients at various weeks after transplantation

rapid engrafting (McCulloch et al., 1964); Trentin, (1978) or the triggering of certain feedback mechanisms by which factors (Burke et al., 1973) are released that stimulate the growth of the transplanted cells. This principle, identified in a rodent model, and successfully applied in clinical marrow transplantation might not only be useful to reduce incidence of leukemia recurrence and improve the kinetics of engraftment, it might if rationally employed be utilized to reduce the very high doses of Cy at present necessary to achieve a take thus increasing the therapeutic index of this drug for transplant conditioning.

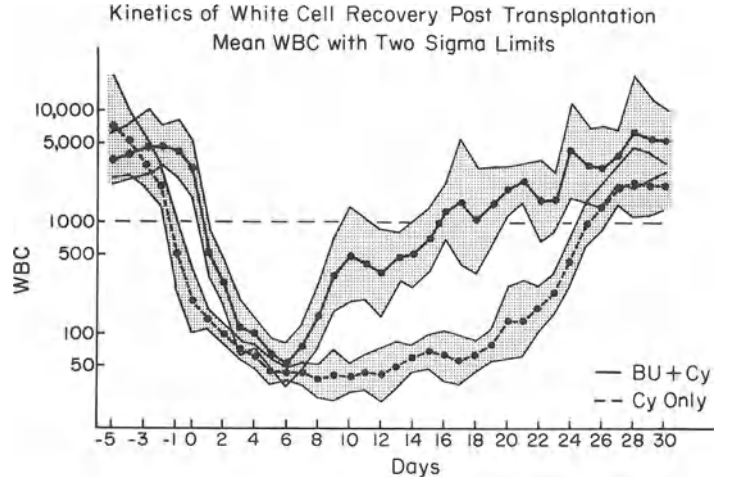


Fig. 3. Peripheral white cell counts in leukemic patients conditioned with Cy and Cy + BU at various days after transplantation

References

1. Burke, P. J., Diggs, C. H., Owens, A. H., Jr.: Factors in human serum affecting the proliferation of normal and leukemic cells. Cancer Res. *33,* 800–802 (1973)
2. Dexter, T. M., Spooncer, E.: Transplantation of cultured hemopoietic stem cells. This vol. pp. 11–18
3. Ford, C. E., Micklem, H. S., Evans, P. E., Gray, J. G., Ogden, B. A.: The inflow of bone marrow cells to the thymus: Studies with part body irradiated mice injected with chromosome marked bone marrow and subjected to antigenic stimulation. Annals N. Y. Academy Science *129,* A.R.T. 283–296 (1966)
4. Humphrey, R. L., Santos, G. W.: Serum protein allotype markers in certain inbred rat strains. Fed. Proc. *20,* 314 (1971)
5. Kolb, H. J., Thierfelder, S., Baumann, P.: Engraftment of allogeneic hemapoietic cells in mice conditioned with non-lethal pretreatment: Antilymphocyte serum (ALS) and partial body irradiation. Exp. Hematol. *22,* 33–34 (1972)
6. McCulloch, E. A., Siminovitch, L., Till, J. E.: Spleen colony formation in anemic mice of genotype W/W^v. Science *144,* 844 (1964)
7. Santos, G. W., Owens, A. H., Jr.: Production of graft-versus-host disease in the rat and its treatment with cytotoxic agents. Nature *210,* 139–140 (1966)
8. Santos, G. W., Owens, A. H., Jr.: Syngeneic and allogeneic marrow transplants in the cyclophosphamide pretreated rat. In: Dausset, I., Hamberger, I., Mathe, G. (Eds.): Advance in Transplantation. Proceedings of the First International Congress of the Transplantation Society, Paris, 27–30 June 1976, pp. 431–436. Copenhagen: Munksgaard 1968
9. Santos, G. W., Burke, P. J., Sensenbrenner, L. L., Owens, A. H., Jr.: Rationale for the use of cyclophosphamide as an immunosuppressant for marrow transplants in man. In: Bertelli, A., Monaco, A. P. (Eds.): Proceedings of the International Symposium on Pharmacological Treatment in Organ and Tissue Transplantation, Vol. 197, pp. 24–31. Amsterdam: Exerpta Medica Foundation 1970
10. Santos, G. W., Sensenbrenner, L. L., Burke, P. J., Mullins, G. M., Anderson, P. N., Tutschka, P. J., Braine, H. G., Davis, T. E., Humphrey, R. L., Abeloff, M. D., Bias, W. B., Borgaonkar, D. S., Slavin, R. E.: Allogeneic marrow grafts in man using cyclophosphamide. Transplant. Proc. *6,* (No. 4), 345–348 (1974)
11. Santos, G. W., Tutschka, P. J.: The effect of busulfan on antibody production and skin allograft survival in the rat. J. Natl. Cancer Inst. *53,* 1775–1780 (1974a)
12. Santos, G. W., Tuschka, P. J.: Marrow transplantation in the busulfan treated rat – A preclinical model of aplastic anemia. J. Natl. Cancer Inst. *53,* 1781–1785 (1974b)
13. Slavin, S., Strober, S.: Mechanisms of specific transplantation tolerance in bone marrow chimeras following total lymphoid irradiation. This vol. pp. 323–331
14. Storb, R., Thomas, E. D., Weiden, P. L., Buckner, C. D., Clift, R. A., Fefer, A., Goodell, B. W., Johnson, F. L., Neiman, P. E., Sanders, J. E., Singer, J.: One hundred ten patients with aplastic anemia (AA) treated by marrow transplantation in Seattle. Transplant. Proc. *10,* 135–137 (1978)
15. Trentin, J. J.: Hemopoietic microenvironments. Transplant. Proc. *10,* (No. 1), 77–82 (1978)
16. Tutschka, P. J., Santos, G. W.: Ag-B incompatible bone marrow transplantation in the rat after treatment with cyclophosphamide (CY) and busulfan (BU). Fed. Proc. *32,* 226 (1973)
17. Tutschka, P. J., Santos, G. W.: Marrow transplantation in the busulfan-treated rat. I. Effect of cyclophosphamide and rabbit anti-rat thymocyte serum as immunosuppression. Transplantation *20,* 101–106 (1975a)
18. Tutschka, P. J., Santos, G. W.: Bone marrow transplantation in the busulfan-treated rat. II. Effect of cyclophosphamide and antithymic serum on the presensitized state. Transplantation *20,* 116–122 (1975b)
19. Tutschka, P. J., Santos, G. W.: Bone marrow transplantation in the busulfan treated rat. III. Relationship between myelosuppression and immunosuppression for conditioning bone marrow recipients. Transplantation *24,* (1), 52–62 (1977)

Colony Forming Units in Culture in Childhood Aplastic Anemia

J. de Koning, E. T. van't Veer-Korthof and M. H. van Weel-Sipman

A. Summary

The results of the determination of the numbers of colony forming units in culture of the bone marrow of 17 children with aplastic anaemia before and after bone marrow transplantation, of 4 children treated with antilymphocyte globulin and of 16 children treated conventionally are presented.

In the aplastic phase the number of C.F.U.-C. was very low to zero. After successful transplantation the number of bone marrow C.F.U.-C. rose but did not become normal. After antilymphocyte globulin and conventional therapy the number of bone marrow C.F.U.-C. remained low, even when a restoration of the haematological values in the peripheral blood took place.

B. Introduction

Aplastic anaemia represents a group of diseases, characterized by severe pancytopenia and a decreased number of granulocytic, erythroid and megakaryocytic cells and their precursors in the bone marrow.

In 37 children with aplastic anaemia the capacity of the bone marrow to form granulocytic colonies in vitro was investigated longitudinally by way of the colony forming units in culture (C.F.U.-C.) technique. Those children were treated either with anabolic steroids (16 patients) or with bone marrow transplantation (17 patients) or with a course of antilymphocyte globulin (4 patients). The methods for clinical bone marrow transplantation and the results in several patients were published more extensively elsewhere (Dooren et al., 1974a; Dooren et al., 1974b., De Koning et al., 1975; Dooren et al., 1976).

The relation between the clinical course, the haematological data and the in vitro colony forming capacity of the bone marrow was studied.

C. Patients

Until February 1979 17 children with severe aplastic anaemia were transplanted with bone marrow cells from HLA identical, mixed lymphocyte culture negative siblings. The group of patients consisted of 11 boys and 6 girls with an age range from 1.5 to 12 years. Twelve patients suffered from idiopathic aplastic anaemia, 3 patients from Fanconi's anaemia, and 1 patient from aplastic anaemia following hepatitis. Bone marrow transplantation was performed in strict protective isolation using laminar down flow isolators and after control of the endogenous microflora by gastrointestinal decontamination. The conditioning of the patients consisted either of cyclophosphamide (4×50 mg/kg body

weight i.v.) alone or a combination of procarbazin (3×12.5 mg/kg body weight orally), antilymphocyte globulin (rabbit anti human lymphocyte globulin, 3×2 mg/kg body weight i.v.) and cyclophosphamide (4×50 mg/kg body weight i.v.). The number of nucleated bone marrow cells administered intravenously ranged from 1.1 to 4.0×10^8/kg body weight. Fourteen patients received one transplant, 3 patients received a second transplant because the first transplantation did not lead to a stable take with haematological recovery. Fourteen patients showed good engraftment of the bone marrow cells. Nine patients had a complete haematological reconstitution and are now 2 to 64 months post-transplantation with a good clinical condition and recovery of the haematological and immunological functions. In this surviving group only 1 patient suffered from a transient subacute graft versus host disease. Eight patients died, 13 days to 8 months following transplantation of complications such as severe graft versus host disease, cardiotoxicity, septicaemia, obstructive bronchiolitis and unexplained acute renal insufficiency. The transplantation data are summarized in the Tables 1 and 2.

Table 1. Transplantation of bone marrow in children with severe aplastic anaemia

No.	Patient	Age	Aetiology	Take	GVHD	†	
1	♀ D.J.	10 yr	Idiopathic	−	−	34 d.	Sepsis
2	♂ J.E.	10 yr	Fanconi	+	++	40 d.	GVHD
7a	♀ P.M.	7 yr	Idiopathic	±?	+		
b				±?	+	25 d.	Cardiotoxicity, wasting, CVA?, sepsis?[a]
8a	♂ G.R.	12 yr	Idiopathic	−	−		
b				−	−	13 d.	Cardiotoxicity, CVA?, sepsis?[a]
9	♂ H.L.	5 yr	Idiopathic	+	+	8 months	Obstructive bronchiolotis
13	♀ W.H.	12 yr	Fanconi	+	++	38 d.	GVHD
14	♂ T.D.	12 yr	Idiopathic	+	−	58 d.	Renal insuff.
15	♂ A.K.	16 yr	Familial	+	++	72 d.	GVHD, CVA

[a] no autopsy performed
CVA: Cerebro-vascular accident

Table 2. Transplantation of bone marrow in children with severe aplastic anaemia

No.	Patient	Age	Aetiology	Take	GVHD	Follow-up months
3	♂ P.v.D.	10 yr	Idiopathic	+	−	64
4a	♂ R.v.K.	6 yr	Idiopathic	±	−	
b				+	−	57
5	♂ H.H.M.	9 yr	Hepatitis	+	−	52
6	♀ A.D.	7 yr	Idiopathic	+	+	49
10	♀ F.K.	12 yr	Fanconi	+	−	37
11	♂ A.v.d.K.	11 yr	Idiopathic	+	−	30
12	♀ P.S.	1.5 yr	Idiopathic	+	−	23
16	♂ P.N.	10 yr	Idiopathic	+	−	7
17	♂ A.P.	10 yr	Idiopathic	+	−	2

A second group of 4 children with severe aplastic anaemia was treated with antilymphocyte globulin, apart from anabolic steroids and supportive care. Three patients received horse anti human lymphocyte globulin (115–160 mg/kg body weight, total dose) and 1 patient received rabbit anti human lymphocyte globulin (10 mg/kg body weight, total dose). Two of these patients also received a transplantation of parental bone marrow cells, 2.35×10^8 and 2.86×10^8/kg body weight respectively. The haematological findings of 1 patient, who did not receive parental bone marrow, improved gradually and she is now recovered several years after antilymphocyte globulin therapy. Another patient did not respond to this treatment and 2 patients are within 2 to 4 months after antilymphocyte globulin therapy. Their follow-up is too short to evaluate the possible effect of this therapy.

A third group of 16 children with aplastic anaemia received only conventional treatment with anabolic steroids and supportive care. Eight patients remained aplastic, 4 of them died. Eight patients showed a partial recovery with platelet counts above 30×10^9/l, a haemoglobin level above 6 μmol/l and granulocyte counts above 1×10^9/l in peripheral blood.

Normal values of C.F.U.-C. were obtained from bone marrow cultures taken from healthy donors for bone marrow transplantation (2 to 22 years of age) and from children (3 to 11 years of age) under general anaesthesia for surgical intervention. The bone marrow punctures were done after informed parental consent.

D. Agar Culture Technique According to Robinson and Pike (1970)

Twenty ml of blood and 2 to 4 ml of bone marrow were sampled in heparinized Hanks balanced salt solution. Erythrocytes were separated from nucleated blood cells and bone marrow by sedimentation with methylcellulose. The supernatant, containing the nucleated cells was sampled and centrifuged. The cells were resuspended in 1 ml of supernatant and counted.

10^6 nucleated blood cells were mixed with a feeder layer prepared from 3 ml Dulbecco (Osm. 800), 2 ml of serum (1/3 horse serum; 1/3 fetal calf serum; 1/3 trypticase Soybroth) and 5 ml Difco agar. One ml of this mixture was used as the colony stimulating layer. Layers prepared with blood from 2 random donors and from the patient were used in the different tests.

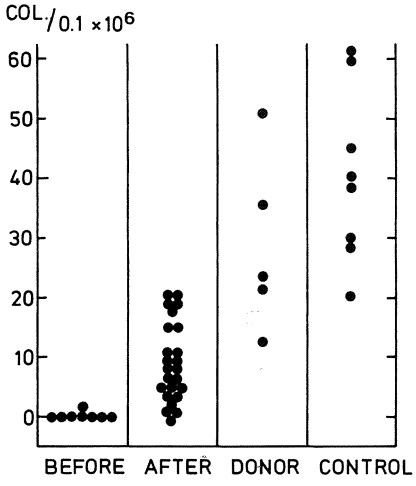
10^5 nucleated bone marrow cells were suspended in 0.3 ml of the heparinized Hanks B.S.S. plus 0.3 ml of a medium consisting of 3 parts Dulbecco, 2 parts of serum and 5 parts of Difco agar. 0.2 ml of the latter suspension was carefully layered over the feeder layer. Nucleated blood cells were also tested for the presence of granulocytic precursors using the same method.

The cultures were done in threefold in Petri dishes, 3.5 cm in diameter, placed in sterile glass containers and incubated at 37.5° C, in 5% carbondioxyde and 100% humidity. After 14 days the number of colonies was counted using a reversed microscope. A colony (C.F.U.-C.) was defined as an aggregate of more than 50 cells assumed to originate from 1 precursor cell. The results of the cultures are given per 0.1×10^6 nucleated cells in bone marrow and blood.

E. Results

The results of the C.F.U.-C. of each bone marrow sample are given in the Figures 1–4.

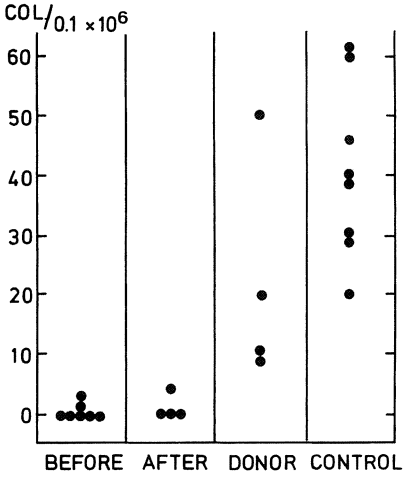
Figure 1 gives the numbers of bone marrow C.F.U.-C. from children with aplastic anaemia, who were successfully treated with bone marrow transplanta-



No. patients	7	9	5	8
No. investig.	8	24	5	8

Fig. 1. C.F.U.-C. in children with aplastic anaemia before and after successful bone marrow transplantation

tion: 7 of the patients were investigated before transplantation and 9 patients on different occasions after transplantation. The results were compared with the numbers of bone marrow C.F.U.-C. from their bone marrow donors and from 8 normal children.

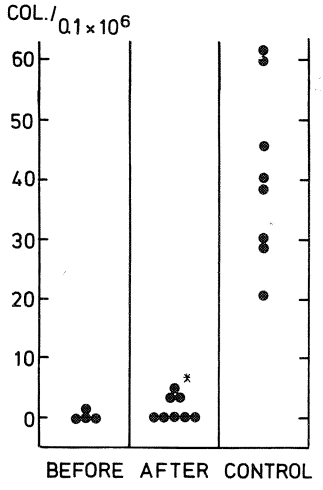


No patients	7	4	4	8
No investig.	7	4	4	8

Fig. 2. C.F.U.-C. in children with aplastic anaemia before and after *un*successful bone marrow transplantation

Figure 2 gives the numbers of bone marrow C.F.U.-C. from children with aplastic anaemia, in whom the bone marrow transplantation was unsuccessful: 7 of the patients were investigated before transplantation and 4 patients after transplantation. The results were compared with the numbers of bone marrow C.F.U.-C. from their bone marrow donors and from 8 normal children.

It is evident that in the aplastic phase of the aplastic anaemia the numbers of C.F.U.-C. are extremely low to zero, compared with the numbers of C.F.U.-C. in patients after successful bone marrow transplantation, in their donors and in normal children. After successful bone marrow transplantation an increase of the numbers of C.F.U.-C. was seen, but these numbers remained low in comparison with those in normal children.



No patients	4	4	8
No investig.	4	8	8

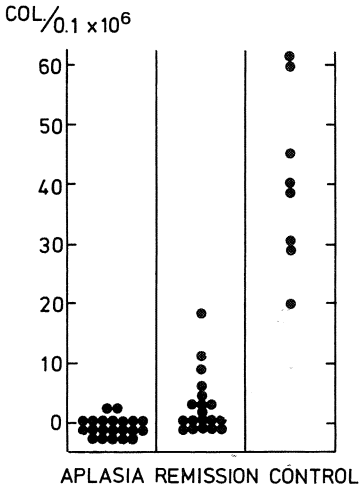
* Haematological Recovery (1 patient)

Fig. 3. C.F.U.-C. in children with aplastic anaemia before and after antilymphocyte globulin treatment

Figure 3 gives the numbers of bone marrow C.F.U.-C. from 4 patients before and after treatment with antilymphocyte globulin: 4 investigations before treatment and 8 investigations thereafter were compared with the numbers of bone marrow C.F.U.-C. from 8 normal children. After antilymphocyte globulin therapy the only patient who showed a complete haematological recovery still had low numbers of C.F.U.-C.

Figure 4 gives the numbers of bone marrow C.F.U.-C. from children with aplastic anaemia who were treated conventionally with anabolic steroids. Twentyone investigations were done in 16 children in the aplastic phase, and 19 investigations were done in 8 patients who reached a partial remission. The results were compared to the numbers of C.F.U.-C. of 8 normal children. Despite a partial haematological recovery during conventional treatment only a very slight increase in the numbers of bone marrow C.F.U.-C. was observed. In all cases the numbers of C.F.U.-C. remained below the range in 8 normal children.

In Figure 5 the numbers of bone marrow. C.F.U.-C. are plotted against the numbers of granulocytes in the peripheral blood of patients before and after successful bone marrow transplantation. For comparison the C.F.U.-C. of the bone marrow and the peripheral blood granulocyte count of 8 normal children are also given. Even when the granulocyte counts in the peripheral blood reached



No patients	16	8	8
No investig.	21	19	8

Fig. 4. C.F.U.-C. in children with aplastic anaemia during conservative treatment

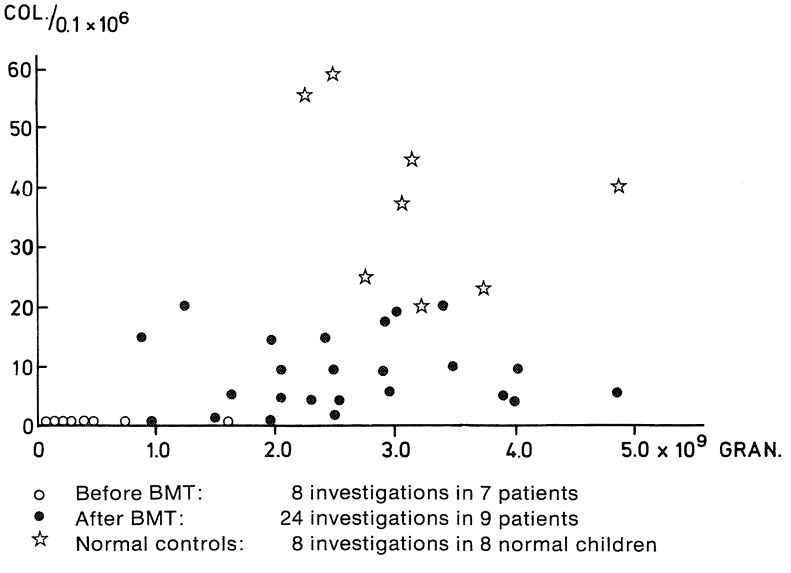


Fig. 5. Peripheral blood granulocytes and bone marrow C.F.U.-C. in children with aplastic anaemia before and after successful bone marrow transplantation compared with normal controls

normal values after haematological recovery, the numbers of bone marrow C.F.U.-C. remained low.

Figure 6 shows the clinical course of 1 patient (P.v.D.) before and after bone marrow transplantation. After bone marrow transplantation no complications were seen. Recently, at 62 months after transplantation, the patient is in a good

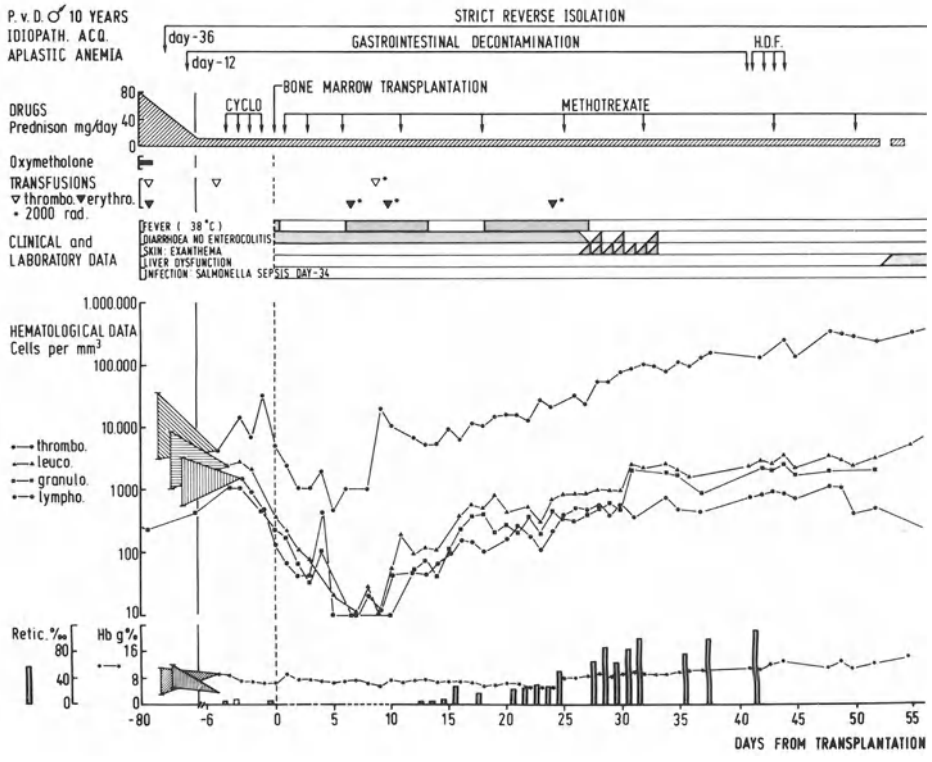


Fig. 6. Clinical course before and after bone marrow transplantation in a patient with low C.F.U.-C. 5 years later

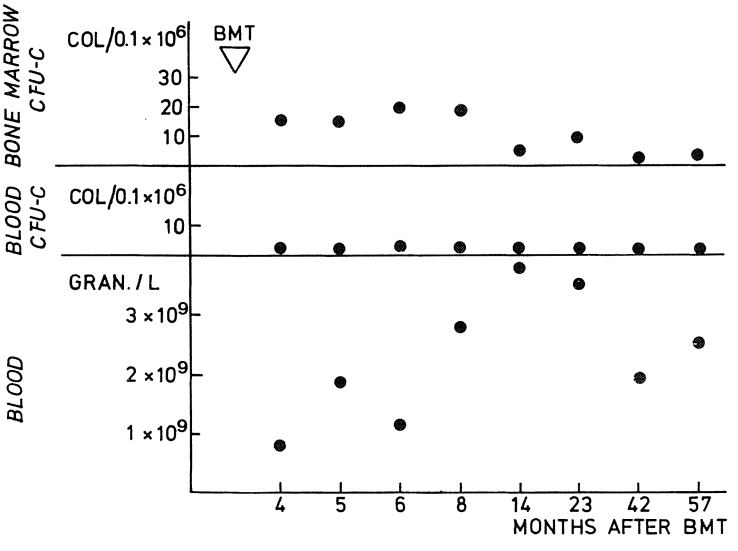


Fig. 7. Peripheral blood granulocytes and C.F.U.-C., and bone marrow C.F.U.-C. after successful bone marrow transplantation in patient P.v.D.

clinical condition with a haemoglobin level of 10 μmol/l, a platelet count of 150×10^9/l and a leucocyte count of 6×10^9/l in the peripheral blood. It is striking (Fig. 7) that a few months after transplantation the numbers of bone marrow C.F.U.-C. reached nearly normal values, but now, more than 5 years after transplantation, these numbers are very low again, despite a normal number of granulocytes in the peripheral blood and a normal bone marrow on cytological examination.

F. Discussion

The observations of an extreme reduction of the number of bone marrow C.F.U.-C. in children with aplastic anaemia in the aplastic phase are in accordance with the findings of others (Kurnick et al., 1971; Dicke et al., 1974; Kern et al., 1977). These low numbers of C.F.U.-C. point to a decreased number of granulocytic precursor cells in the bone marrow aspirates. These decreased numbers of bone marrow C.F.U.-C. from such patients cannot only be explained by a dilution of the aspirated bone marrow cells with blood (Haak, 1977). However, in further experiments it should be tried to incorporate automated quantitative cell identification techniques in order to compare the C.F.U.-C. data with the pattern of samples of nucleated cells brought into the cultures.

The findings of low numbers of bone marrow C.F.U.-C. in children who recovered (partially) from aplastic anaemia after either conservative treatment or antilymphocyte globulin therapy may reflect a still limited pool of precursor cells as the expression of a very slow recovering process in the bone marrow. Friedman et al. (1974) described residual abnormalities on careful haematological examination in 10 long term survivors out of 23 children with severe aplastic anaemia. Although all their surviving patients were asymptomatic and clinically well, these authors consider aplastic anaemia as a disease with a very long course. The slow clinical recovery and the low C.F.U.-C. numbers can point to intrinsic stem cell defects as a cause of aplastic anaemia but also to primary or secondary autoimmune phenomena.

A primary autoimmune phenomenon is assumed by Ascensão et al. (1976) who observed increased cloning efficiency of the C.F.U.-C. in aplastic anaemia by treating the aspirated bone marrow with antilymphocyte globulin. Singer et al. (1976) suggest that the results obtained by Ascensão are elicited by multiple blood transfusions and not due to a primary autoimmune phenomenon.

More striking are the low numbers of bone marrow C.F.U.-C. in the recipient of a successful allogeneic bone marrow transplantation. In agreement with the results of bone marrow transplantation elsewhere (Storb et al., 1977) we saw a complete chimerism in 9 out of 17 transplanted patients. In these 9 children complete chimerism could be proven with sex markers, red blood cell markers and a serum Gm groups. The complete chimerism indicates the outgrowth of transplanted donor stem cells. These donor stem cells are assumed to be free of intrinsic defects, as can be seen in C.F.U.-C. controls. The low numbers of C.F.U.-C. in transplanted patients could be due to the same causes as described above. One could suppose that the pool of cycling precursor cells in the bone

marrow could be sufficient to raise reasonable numbers of granulocytes, erythrocytes and thrombocytes, but still too small to produce normal C.F.U.-C.

Some support of this idea can be found in animal experiments in mice and dogs.

In transplantation of syngeneic bone marrow cells in mice after lethal irradiation Vos (1972) observed a repopulation of the C.F.U. in bone marrow in 10–40 days depending on the dosage of cells, but the repopulation remained on subnormal levels.

Playfair et al. (1965) observed this repopulation as well, much faster in the spleen (C.F.U.-S.) than in the animals' bone marrow.

Nothdurft et al. (1978) followed the C.F.U.-C. in blood and bone marrow of dogs after lethal irradiation and allogeneic transfusion with cryopreserved blood mononuclear cells and observed in some dogs a slow regeneration of bone marrow C.F.U.-C. compared to others, but, still this regeneration was a matter of days rather than weeks or months as found in the human aplastic anaemia transplanted with HLA identical bone marrow. Still these animal experiments do not fully explain the results of the low C.F.U.-C's found in man after transplantation with HLA identical bone marrow cells.

Acknowledgements

The authors wish to thank Prof. D. W. van Bekkum M. D. and B. Löwenberg M. D. of the Radiobiological Institute T.N.O., Rijswijk, The Netherlands, for their technical and organisational help.

The authors are grateful to "Het Koningin Wilhelmina Fonds" (KG 77–53) and the Organisation for Health Research T.N.O. (GO-731-67) for financial support and also want to express their gratitude to Mrs. N. J. Oudeman-Gruber and Mrs. S. Koper-Schmitz for their excellent technical assistance.

References

Ascensão, J., Kagan, W., Moore, M. A. S., Pahwa, R., Hansen, J., Good, R.: Aplastic anaemia: evidence for an immunological mechanism. Lancet *I,* 669–671 (1976)

Dicke, K. A., Löwenberg, B.: In vitro analysis of pancytopenia: its possible relevance to the clinical course and the preleucaemic state in aplastics. K. Lindahl-Kissling, D. Osda (Eds.): Proceedings of the 8th leucocyte culture conference, 417–424. London: Academic Press 1974

Dooren, L. J., Kamphuis, R. P., de Koning, J., Vossen, J. M.: Bone marrow transplantation in children. Semin. Hematol. *11,* 369–382 (1974)

Dooren, L. J., de Koning, J., Kamphuis, R. P., Uittenbogaart, C. H., Brubakk, A. M., Vossen, J. M.: Knochenmarktransplantation bei Patienten mit aplastischen Anaemie. Blut *28,* 211–217 (1974)

Dooren, L. J., Vossen, J. M., de Koning, J., Kamphuis, R. P.: Bone marrow transplantation in 10 children with aplastic anemia. Clinical results and immunological follow-up. Exp. Hematol. *4,* suppl. p. 150 (1976)

Freedman, M. H., Saunders, E. F., Hilton, J., McClure, P. D.: Residual abnormalities in acquired aplastic anaemia of childhood. J. Amer. Med. Ass. *228,* 201–202 (1974)

Haak, M. L.: Acquired aplastic anaemia in adults. Leiden: Thesis 1978

Kern, P., Heimpel, H., Heit, W., Kubanek, W.: Granulocyte progenitor cells in aplastic anaemia. Br. J. Haematol. *35,* 613–623 (1977)

Koning, J. de, Vossen, J. M., Dooren, L. J., Kamphuis, R. P.: Beenmergtransplantatie. Natuur en Techniek *43*, 379–388 (1975)
Kurnick, J. E., Robinson, W. A., Dickey, C. A.: In vitro granulocytic colony forming potential of bone marrow from patients with granulocytopenia and aplastic anemia. Proc. Soc. Exp. Biol. Med. *137*, 917–921 (1971)
Nothdurft, W., Fliedner, Th. M., Calvo, W., Flad, H. D., Huget, R., Körbling, M., Krumbacher-von Loringhofen, K., Ross, W. M., Schnappauf, H. P., Steinbach, I.: CFU-C populations in blood and bone marrow of dogs after lethal irradiation and allogeneic transfusion with cryopreserved blood mononuclear cells. Scand. J. Haematol. *21*, 115–130 (1978)
Playfair, J. H. L., Cole, L. J.: Quantitative studies on colony-forming units in isogenic radiation chimeras. J. Cell Comp. Physiol. *65*, 7–18 (1965)
Robinson, W. A., Pike, B. E.: Colony growth of human bone marrow cells in vitro. Hemopoietic cellular proliferation, 249–259. New York: Grune & Stratton 1970
Singer, J. W., Brown, J. E., James, M. C., Doney, K., Warren, R. P., Storb, R., Thomas, E. D.: Effect of peripheral blood lymphocytes from patients with aplastic anemia on granulocytic colony growth from HLA matched and mismatched marrows: effect of transfusion sensitization. Blood *52*, 37–45 (1978)
Storb, R., Ross, L., Thomas, E. D.: Treatment of aplastic anemia by marrow transplantation from HLA identical siblings. J. Clin. Invest. *59*, 625–632 (1977)
Vos, O.: Multiplication of haemopoietic colony forming units (CFU) in mice after X-irradiation and bone marrow transplantation. Cell. Tissue Kinet. *5*, 341–350 (1972)

Discussion

Heimpel: We followed the group of patients which we have published in 1977 and these patients came into complete remission with normal blood values. In these patients we saw a tendency of increase in CFU-C values, we never saw values of zero. Where do the granulocytes in your patients come from if not from CFU-C? What do you call remission?
de Koning: The remission of our conventionally treated patients was partial, platelet levels were never above 150,000, hemoglobin was normal in some cases and marrow was hypocellular. This correlates with low CFU-C levels. For the low values in the marrow grafted group I have no good explanation. We as others have not been able to reproduce the incubation experiment of Ascea.
Dicke: It may be worthwile to study your patients with the Dexter technique in order to see whether you have the right stimulus for colony growth.
de Koning: The stimulus was alright, since we used leukocytes of normal donors as underlayer. I know from experiments of others that also patient leukocytes stimulate colony growth as underlayer. So the real defect is in the stem cell.
Dicke: You should use the donor's leukocytes and not completeley allogeneic leukocytes.
Storb: Did you try to take T-cells out of your cultures by rosetting instead of ATS?
de Koning: No.

Split Chimerism in Three Patients Suffering from Severe Combined Immunodeficiency (SCID)*

D. Niethammer, S. F. Goldmann, H. D. Flad, P. Wernet, G. Stursberg, J. Colombani, T. M. Fliedner, and E. Kleihauer

A. Summary

Three children, suffering from SCID, were lacking a genotypically identical sibling. One of them demonstrated spontaneous graft-versus-host (GvH) reaction due to a maternofetal transfusion. They were transplanted with the marrow of the HLA-A-mismatched but otherwise identical mother (1), with that of the completely mismatched mother in the case of spontaneous GvH (2) and with cultivated thymus epithelium (3). Full (twice) or partial (once) immunoreconstitution was achieved. Total bone marrow or myeloid suppression was seen in all three cases, as well as autoimmune hemolytic anemia in one case.

In the patient who received thymus epithelium, engraftment of donor derived T-cells was detected. Further maturation of the host's own B-cells was observed and a certain amount of cooperation developed between the completely HLA-mismatched donor derived T-cells and the host's B-cells.

B. Introduction

SCID is a disease which regularly leads to death during the first year of life. Transplantation of bone marrow of a histocompatible sibling is the treatment of choice [1]. Using this procedure the chances of complete reconstitution of the immune function are high. However, quite often a genotypically identical donor is not available due to inheritance of the disease and the small number of children in contemporary families. Experience is still quite limited with alternative therapeutical approaches [2]. Donors other than HLA-genotypically identical siblings have been used [3]. Bone marrow has been transplanted from related donors, who were HLA-genotypically or phenotypically identical or who were HLA-D-compatible but mismatched in the HLA-A and/or HLA-B loci [4–9]. Graft-versus-host disease (GvH) was in general more severe and was frequently associated with bone marrow suppression or aplasia [10]. Sometimes only engraftment of the T-cells was detectable [5]. Twice unrelated donors were used for bone marrow transplantation [11, 12].

Fetal liver was also used as the source for stem cells (reviewed in [2]). 6 of 21 cases achieved durable T-cell reconstitution, some of them also showed evidence

* Supported by Deutsche Forschungsgemeinschaft (SFB 112) and DFG-Forschergruppe "Leukämieforschung", Wa 139/10

of some B-cell function. In most successfully transplanted cases a fetal thymus was given in addition.

The transplantation of fetal thymus alone was without success in a great number of patients with SCID [2]. In some cases, however, engraftment of the T-cells could be achieved [13–17]. Sometimes the thymus may only provide inductive influences to endogenous lymphoid precursors that are deficient without any evidence of engraftment [2]. Only a certain kind of patients with SCID may respond in this way concluded from experimental evidence. They would be the same patients who would respond to the transplantation of cultivated thymus [18]. On the same line thymosin may induce immune function in immunodeficient patients [19].

Patients with SCID are normally not conditioned before transplantation. This is why the development of split chimerism is quite common, meaning, that only the lymphocytes or only the T-cells are engrafted. This has been observed after transplantation of bone marrow, fetal liver or fetal thymus, but not after transplantation of cultivated thymus.

Here we report about three patients suffering from SCID, who developed split chimerism after the transplantation of maternal marrow or cultivated thymus. Besides GvH in all three cases, we saw evidence of partial or complete bone marrow suppression in all three patients and autoimmune hemolytic anemia in one patient. Some aspects of this paper have already been presented elsewhere [20].

C. Methods

All patients were kept in a plastic isolation system [21]. Unless stated otherwise routine laboratory methods were used. HLA-ABC typing was done by the complement dependant lymphocytoxic test [22]. HLA-DR (B-cell) typing was performed using both, the two colour fluorescence test [23] and the workshop standard method [24]. The mixed lymphocyte culture (MCL) microtechnique employed for direct MLC and HLA-D typing is described elsewhere [9, 25]. Receptors for IgM, Fc and Ia were determined using an indirect immunofluorescence technique [26, 27]. The determination of T-cell rosettes and complement receptors (C4b and C3d) was described previously [27, 28].

D. Case reports and results

Case 1 (M.W.): The clinical course of the first patient has already been described in detail [9, 29]. In short: The first child of the parents, a boy, had died from SCID at the age of seven months. Our patient, also a boy, was delivered by caesarian section and placed immediately after birth into the isolation system. The diagnosis of SCID with elevated B-cells was confirmed (Table 1). No sibling was available and at the age of 9 months he was transplanted with the marrow of the MLC-negative (compatible) mother. There was a mismatch in one HLA-A-antigen and the rhesus antigens (Table 2). Severe acute GvH developed which was treated with steroids and antithymocyte globulin. He survived the acute GvH probably due to the germfreeness. The acute phase with severe involvement of the skin, liver and gut was followed by chronic GvH with repeated episodes of

Table 1. Evidence of SCID in the three patients

	M.W. ♂	M.K. ♀	M.B. ♂
Family history of SCID	1 brother	1 brother, 2 sisters	1 brother
Adenosine deaminase	+	+	+
Thymus shadow	Ø	Ø	Ø
Lymphocytes	normal	low (180–340/μl)	normal
T-cells			
AET–RFC	2–21%	31%	8%
Reponse to PHA	Ø	Ø	Ø
Con A	Ø	Ø	Ø
allog.cells	Ø	Ø	(+)
DNCB-test	Ø	Ø	Ø
B-cells			
EAC–RFC	70–85%	40%	23%
Immunoglobulins	Ø	Ø	Ø
Isohemagglutinins	Ø	Ø	Ø
Antibody resp. to vacc.			
Polio	Ø	Ø	Ø
Diphtheria	Ø	Ø	Ø
Tetanus	Ø	Ø	Ø
Response to			
Pokeweed mitogen	Ø	Ø	Ø

skin rushes and long lasting elevation of the liver enzymes. 150 days after the transplantation the patient was completely immunologically reconstituted (Table 3).

Clear evidence of split chimerism could be shown (Table 2). Only the lymphocytes of the mother were engrafted; the platelets and red cells remained of the child's type. At the time when immunocompetence could be proven, hemolytic anemia developed. This was due to anti-D- and anti-C-antibodies, a consequence of the different rhesus antigens of mother and child. During the following time agranulocytosis developed and nine months after the transplantation the child was completely aplastic. Two other transplantations did not lead to an engraftment of the maternal hematopoiesis and the child died from complications of total bone marrow aplasia [29].

Case 2 (M.K.): Three siblings of the patient (two sisters and one brother) had died from SCID. The girl was born two months early and was immediately placed into the isolation system [30]. Oral decontamination was started. The immunological tests revealed SCID with lymphopenia (Table 1). At the age of two months the lymphocytes increased to normal, and eosinophilia and a skin rush developed. This resembled the symptoms of GvH in spite of the fact that the child had not received a blood transfusion or transplantation. A skin biopsy confirmed our suspicion and GvH was finally proven by the fact that the HLA-antigens of the lymphocytes, which were father derived, had disappeared. They were replaced by those of the mother. The probable cause of the phenomen was a maternofetal transfusion into the immunodeficient patient. No signs of involvement of the liver or gut could be detected. In addition, a B- and T-cell

Table 2. Evidence of chimerism in the three patients

Pat. Donor	Lymphocytes		Platelets		Erythrocytes	
	before Trpl.	after Trpl.	before Trpl.	after Trpl.	before Trpl.	after Trpl.
M.W. *Mother* HLA-A2,28 HLA-B7,14 DW2 Bloodgroups 0 rh-li	HLA-A9,28 HLA-B7,14 DW2	HLA-A2,28 HLA-B7,14 DW2	n.d.	HLA-A9	O Rh+	O Rh+
M.K. *Mother* HLA-A26(10), 29 HLA-B38(16) BW44(12)	HLA-A29, W24(9) HLA-B39(16), W44(12)	HLA-A26(10), 29 HLA-B38(16), W44(12)	HLA-A29,9 HLA-B12, W16	HLA-AW26(10), 29 HLA-B12, Y1(W38?)	B rh Ø	B Rh+
M.B. *Cultivated Thymus* HLA-A2,W24 HLA-B15.2, 17.1 HLA-CW3,W6	*B- and T-cells:* HLA-A2,26 HLA-BW38, W44 HLA-CW2 DRW1,W6	*B-cells:* HLA-A2,26 HLA-BW38, W44 HLA-CW2 DRW1,W6 *T-cells:* HLA-A2,W24 HLA-B15.2, 17.1 HLA-CW3,W6	n.d.	n.d.	B rh −	B rh −

Table 3. Evidence of reconstitution of the immune function in the three patients after transplantation

	M.W.	M.K.	M.B.
Lymphocytes	normal	normal	normal
T-cells			
AET–RFC	65%	70%	20–40%
Response to			
PHA	+++	+++	++
Con A	+++	+++	++
allog.cells	+++	+++	++
B-cells			
EAC–RFC	20%	20%	10–40%
Immunoglobulins	normal	normal	+but low
Antibodies:			
Isohemaggl.	Ø	Ø	Ø
Diphtheria	+++	+++	Ø
Tetanus	+++	+++	Ø/+
Polio	+++	+++	Ø
Response to			
Pokeweed M.	+++	+++	+++

chimerism could not be excluded, since the patient's lymphocytes continued to stimulate those of the mother in the mixed lymphocyte culture (Table 4). Whether mother derived B-cells circulated in parellel to the original B-cells of the host in the peripheral blood of the patient could not be tested since reagents for the HLA-D/DR determinants of the patient's family were not available.

In spite of the increase of the lymphocytes and a transitory slight reactivity to PHA and allogeneic cells no immunocompetence developed. At the age of 11 months severe asthma occurred with increased IgE values and at the age of 12 months agranulocytosis followed. The situation started to resemble that seen in the first patient (split chimerism and agranulocytosis), who ended with aplastic anemia. To avoid further deterioration of the hematological situation, a bone marrow transplantation was anticipated.

Neither an identical sibling nor an MLC-negative related or unrelated donor was available. So it was decided to use the completely mismatched mother (Table 2) as a donor since the transfusion of her lymphocytes had only induced a chronic GvH of the skin. The child was pretreated with cyclophosphamide to achieve a full engraftment of the maternal marrow. The transplantation was performed at the age of 14 months. A short episode of acute GvH occurred. Full engraftment of the maternal hematopoiesis as well as of the lymphopoiesis could be proven (Table 2) and the child became completely immunocompetent (Table 3). The mixed lymphocyte culture between mother and child was now negative (Table 4). At the age of 20 months she was released from the isolation system. Four weeks later she died from sudden infant death.

Table 4. Results of mixed lymphocyte culture of patient 2 (M. K.) and her mother. Data are given in stimulation ratios (x are stimulating cells)

	$Patient_x$	$Mother_x$	$Control_x$
Patient			
at 1 month	1.0	2.1	1.9
at 4 months (before BMT)	1.0	1.9	2.6
at 16 months (after BMT)	1.0	1.1	19.4
Mother			
at 1 month	13.8	1.0	27.6
at 4 months	31.1	1.0	56.3
at 16 months	1.0	1.0	14.0

Case 3 (M.B.): The brother of the third child had died from SCID. This patient, also a boy, was isolated at the age of 5 months because of SCID (Table 1). Again no sibling or other possible donor was available. At the age of 8 months the child was transplanted with cultivated thymus epithelium (cultivated by H. M. Dosch and F. W. Gelfand, Toronto). Ten days later transitory signs of GvH occurred (fever, skin rush, elevation of liver enzymes) which subsided after one week. Repeating of the tissue typing revealed a completely different HLA-phenotype which was identical with that of the 9-month-old donor of the thymus (Table 2). Of course, no evidence of engraftment of hemopoietic cells could be detected. Two weeks later the HLA-phenotype of the donor had disappeared again. During the following time the T-cells increased to values between 20 and 40% and slowly started to respond to mitogens and allogeneic cells. Repeated tissue typing of

Table 5. Cell surface markers in two patients with SCID. In addition amount of T-cells and serum-IgM values are given. (BMT = bone marrow transplantation, CTT = cultivated thymus transplantation)

Patient	I a %	Fc %	IgM %	C 4 b %	C 3 d %	T-rosettes %	Serum-IgM mg%
1 (M. W.) before BMT	95	48	41	37	32	6	∅
3 (M. B.) before CTT	31	68	3	6	38	2	∅
After CTT	82	49	50	41	29	15	20
Control	19	17	12	18	17	81	n.d.

enriched B- and T-cells revealed two different allogeneic populations (Table 2): The B-cells carried the phenotype of the host, the T-cells that of the donor. Determination of the surface markers of the lymphocytes before the transplantation had revealed a pattern of two populations, with different receptors (Table 5). One population apparently carried only FC receptors. The other carried receptors for Ia-like antigen and C3d. Almost no receptors could be seen for IgM and C4b. After the transplantation of the cultivated thymus the situation changed. 80% of the cells now carried Ia-receptors. And half of the cells had receptors for IgM and C4b. The pattern was identical with that of patient 1 (MW) (Table 5). The T-cells had increased at that time to 15% and about 20 mg% of IgM was detectable in the serum. At the same time no thymus derived B-cells were found. During the following months IgA, IgG and IgM were detected in changing amounts. Repeated vaccinations over a period of one year did not lead to specific antibody formation for poliomyelitis, diphtheria and tetanus. Now, more than one year after the transplantation, a positive titer for tetanus was detected which increased after an additional vaccination.

At present – 14 months after the transplantation of the cultivated thymus – the T-cell numbers are still not normal but the response to mitogens and allogeneic cells is high. Two months after the transplantation, neutropenia started which is still present (<500 neutrophils/μl). This is why the child is still in the isolation system thriving normal without any evidence of infection.

E. Discussion

Transplantation of bone marrow from a genotypically identical sibling normally restores the immune function of the T- and B-cells in patients suffering from SCID [1]. In these three cases no such donor was available and an alternative way had to be discussed. Table 6 summarizes the data of our three patients pertaining the transplantation. In the first child the HLA-A mismatched but MLC-identical mother seemed to be a good choice. The second child hat spontaneous moderate GvH of the skin due to maternofetal transfusion. In this case agranulocytosis developed, which made a bone marrow transplantation necessary to restore the myelopoesis. Due to the unknown HLA-D/DR-determinants of the family a search for an unrelated donor was impossible, since we had no original B-cells of the child. In the third case we had used the cultivated thymus epithelium, because

Table 6. Summary of the facts, pertaining the transplantation of the three patients

Patient	Donor	Engraftment	GvH-reaction	Immunological reconstitution	"Immunological" complications
1 (M.W.)	HLA-A mismatched mother	T-cells B-cells no hemopoesis	severe acute + chronic	T- and B-cell function	Immune hemolytic anemia, Aplastic anemia
2 (M.K.)	HLA-A, -B and -D mismatched mother	*1. spontaneous* T-cells (B-cells?) *2. after Cyclophosphamide* T-cells B-cells hemopoesis	mild chronic moderate acute, mild chronic	none T- and B-cell function	Agranulocytosis (Sudden infant death?)
3 (M.B.)	cultivated thymus epithelium (unrelated mismatched child)	only T-cells	mild to moderate acute, no chronic	T-cell function, no specific B-cell function (?)	Neutropenia

the patient's lymphocyte function normalized under the influence of thymosin (unpublished results).

In aplastic anemia or leukemia the full engraftment of the hemopoiesis as well as of the lymphopoiesis is the result of transplantation. In SCID using a compatible sibling as the donor only engraftment of the T- and B-cells normally is achieved. This was true for our first patient and the second patient after treatment with cyclophosphamide and it had probably already happened spontaneously. Engraftment of the T-cells alone is the rule after successful transplantation of fetal thymus alone and in most cases, who have been transplanted with fetal liver [2]. Engraftment, particularly of B-lymphocyte precursors, had been inconsistent and difficult to achieve, when marrow of HLA-phenotypically identical or HLA-D-compatible related or unrelated donors has been used (3 of 10 cases) (Table 7). In our third patient, to our surprise, T-cells from the cultivated thymus epithelium were engrafted. This has not been observed before and had not been the aim of the transplantation. Surprisingly enough these cells did not cause either severe acute or chronic GvH.

The incidence of GvH seems to be more frequent and the manifestations more severe in those cases, where an identical sibling was not used [1]. Nevertheless, judged from the data given in Table 7, there is no consistant pattern when the incidence and severity of GvH is compared with the degree of HLA-incompatibility. Only mild or moderate acute and chronic GvH was observed after the engraftment of completely incompatible marrow [30] or of the T-cells from the incompatible cultivated thymus. So other antigens than HLA-antigens must be important for the incidence and the severity of GvH. This

Table 7. Lymphoid Reconstitution of SCID following transplantation of marrow from donors other than genotypically identical siblings (according to (2) and completed)

Pat.	Donor	Degree of HLA-compatibility	HLA-incompatibilities	Engraftment of donor cells	GVH	Reconstitution of		Ref.
						T-cells	B-cells	
1	Uncle	Genotypic	None	T-cells	None	+(donor)	+(host)	6
2	Father	Genotypic	None	T-cells	Mild	+	−	8
3	Father	Phenotypic	None	T- and B-cells	Severe	+	+ (delayed)	7
4	Father	Phenotypic	None	T- and B-cells	Moderate	+	+	2
5	Uncle	Nonidentical	2 B-Antigens	T-cells	Moderate	+	−	5
6[a]	Sibling	Nonidentical	1 B-Antigen	T- and B-cells	Moderate	+	+	4
7[a]	Mother	Nonidentical	1 A-Antigen	T- and B-cells	Severe	+	+	9
8	Father	Nonidentical	1 A-Antigen	T- and B-cells	None	+	+	2
9[a]	Unrelated	Nonidentical	1 A-Antigen	T- and B-cells	Moderate	+	+	12
10[b]	Mother	Nonidentical	1 A-Antigen 1 B-Antigen 1 D-Antigen	T- and B-cells	Moderate	+	+	30

[a] Patients with aplastic anemia as complication after transplantation
[b] Patient with agranulocytosis as complication after maternofetal transfusion

is already suggested by the fact that GvH even in fully matched sibling pairs cannot be predicted. Engraftment against the MLC-locus has not been achieved in patients with SCID up to now [1, 2]. This patient (M.K.) is also the first child who has been transplanted because of the occurrence of "spontaneous" GvH after maternofetal transfer of lymphocytes with the marrow of the mother.

Interaction between T- and B-cells has been mainly studied in experimental models. It has been shown that cooperation is more efficient between histocompatible lymphocytes [31]. This seems to be also true in man. Supporting evidence was derived from two patients who were transplanted with an HLA-genotypically identical uncle [6] or sibling [17]. In both cases only the T-cells were engrafted. Full cooperation was observed, since the B-cell function was completely restored. On the other hand, no B-cell function was observed in those patients, whose T-cell function had recovered after engraftment of T-cells derived from fetal liver and/or thymus [2, 17]. Our patient, having received a transplantation of cultivated thymus epithelium responded immediately with the production of IgM and IgA. IgG followed much later and the levels of the serum immunoglobulins were low after an initial rise, but they were not negative again. Repeated vaccinations over a period of one year did not induce a specific antibody response. Now more than a year after the transplantation the child started to produce tetanus antibodies, suggesting a cooperation between the HLA-mismatched thymus derived T-cells and the host derived B-cells. Of course it cannot be fully excluded that there is a small population of donor derived B-cells which is not detectable by histocompatibility typing. Such cells would simulate a cooperation. But it is most unlikely, that the thymus epithelium contained stem cells with a proliferative capacity for B-cells.

On the other hand, there can be no doubt, that the transplantation induced some kind of maturation of the host's B-cells. This could be concluded from the

changes of the surface receptors (Table 5). A marked increase of the already existing Ia-receptors and the appearance of IgM- and C4b receptors could be detected. After the transplantation the values were comparable to those of the child (M.W.), who had suffered from SCID with high B-cells. It apparently had already B-lymphocytes, whose maturation was more advanced, judged from the receptors present on their surface. Ia-like antigens probably distinguish most accurately the B-cell population from the other types of lymphocytes [32]. The findings also suggest, that in both cases a faulty maturation occurred which led to a high percentage of receptors for Ia-like antigen, Fc, IgM and complement.

Unexpected was the high incidence of bone marrow aplasia or myeloid suppression one of which was found in all three patients. The cause of this is unknown but the relationship to the transplantation or maternofetal transfusion cannot be doubted. The most likely cause is some kind of an "autoimmune" phenomen, due to the split chimerism.

In Table 7 ten patients from the literature are listed who received bone marrow from donors other than genotypically identical sibling. In four out of six patients who were transplanted with nonidentical marrow signs of bone marrow suppression occurred, which was overcome in three cases with a new transplantation of bone marrow from the same donor without [4] or with [12, 30] pretreatment with cyclophosphamide. In these four cases like in our patient who received the cultivated thymus transplant, engraftment of T-cells had occurred before the incidence of bone marrow suppression.

Treatment of patients with SCID lacking a genotypically identical sibling remains a challenge. The likelihood to encounter other and still unknown problems is great.

References

1. Bortin, M. M., Rimm, A. A.: For the Advisory Committee of the International Bone marrow Transplant Registry. Severe combined immunodeficiency. Characterization of the disease and results of transplantation. JAMA *238,* 591 (1977)
2. O'Reilly, R. J., Pahwa, R., Dupont, B., Good, R. A.: Severe combined immunodeficiency: Transplantation approaches for patients lacking an HLA genotypically identical sibling. Transplant. Proc. *10,* 187 (1978)
3. Dupont, B.: Histocompatibility testing for clinical bone marrow transplantation and prospects for identification of donors other than HLA genotypically identical siblings. This vol. pp. 121–134
4. Meuwissen, H. J., Gatti, R. A., Teresaki, P. I., Hong, R., Good, R. A.: Treatment of lymphopenic hypogammaglobulinemia and bone-marrow aplasia by transplantation of allogeneic marrow. N. Engl. J. Med. *281,* 691 (1969)
5. Copenhagen Study Group of Immunodeficiency: Bone marrow transplantation from an HLA nonidentical but mixed-lymphocyte-culture identical donor. Lancet *I,* 1146 (1973)
6. Vossen, J. M., de Koning, J., van Bekkum, D. W., Dicke, K. A., Eysvoogel, V. P., Hijmans, W., van Loghem, E., Rádl, J., van Rood, J. J., van der Waay, D., Dooren, L. J.: Successful treatment of an infant with severe combined immunodeficiency by transplantation of bone marrow cells from an uncle. Clin. Exp. Immunol. *13,* 9 (1973)
7. Anderson, J. M.: Bone marrow transplantation. Proc. R. Soc. Med. *68,* 577 (1975)
8. Geha, R. S., Malakian, A., Le Franc, G., Chayban, D., Serre, J.-L.: Immunological reconstitution in severe combined immunodeficiency following transplantation with parental bone marrow. Pediatrics *58,* 451 (1976)

9. Niethammer, D., Goldmann, S. F., Haas, R. J., Dietrich, M., Flad, H.-D., Fliedner, Th. M., Kleihauer, E.: Bone marrow transplantation for severe combined immunodeficiency with the HLA-A incompatible but MLC-identical mother as a donor. Transplant. Proc. *8*, 623 (1976)
10. Dupont, B., Hansen, J. A., Good, R. A., O'Reilly, R.: Histocompatibility testing for clinical bone marrow transplantation. In: Ferrara, G. B. (Ed): HLA-System-New Aspects, p. 153. Elsevier North-Holland Biomedical Press 1977
11. Horowitz, S. D., Bach, F. H., Groshong, T.: Treatment of severe combined immunodeficiency with the bone marrow from an unrelated mixed-leukocyte-culture-non-reactive donor. Lancet *II*, 431 (1975)
12. O'Reilly, R. J., Dupont, B., Pahwa, S., Greimes, E., Smithwick, E. M., Pahwa, R., Schwartz, S., Hansen, J. A., Siegal, F. P., Sorell, M., Sveygaard, A., Jersild, C., Thomsen, M., Platz, P., L'Esperance, P., Good, R. A.: Reconstitution in severe combined immunodeficiency by transplantation of marrow from an unrelated donor. N. Engl. J. Med. *297*, 1311 (1977)
13. Githens, J. H., Fulginiti, V. A., Suvatte, V., Schroth, G., Hathaway, W. E., Tellmann, D. F., Kay, H. E. M., Teresaki, P. I., Hill, G. J., Kempe, C. H., Cox, S. T.: Grafting of fetal thymus or hemopoietic tissue in infants with immunodeficiency syndromes. Transplantation *15*, 427 (1973)
14. Ammann, A. J., Wara, D. W., Salmon, S., Perkins, H.: Permanent reconstitution of cellular immunity in a patient with sex-linked combined immunodeficiency. N. Engl. J. Med. *289*, 5 (1973)
15. Rachelefsky, G. S., Stiehm, E. R., Ammann, A. J., Cederbaum, S. D., Opelz, G., Teresaki, P. I.: T-cell reconstitution by thymus transplantation and transfer factor in severe combined immunodeficiency. Pediatrics *55*, 114 (1975)
16. Haneberg, B., Fronland, S. S., Finne, P. H., Bakke, T., Thunold, S., Mol, T. I., Tónder, O., Solberg, C. O., Solheim, B. G., Dahlem, A.: Fetal thymus transplantation in severe combined immunodeficiency disease. Scand. J. Immunol. *5*, 917 (1976)
17. Griscelli, C., Durandy, A., Ballet, J. J., Prieur, A. M., Hors, J.: T- and B-cell chimerism in two patients with severe combined immunodeficiency (SCID) after transplantation. Transplant. Proc. *9*, 171 (1977)
18. Hong, R., Schulte-Wissermann, H., Horowitz, S., Borzy, M., Finlay, J.: Cultured thymic epithelium (CTE) in severe combined immunodeficiency. Transplant. Proc. *10*, 201 (1978)
19. Wara, D. W., Ammann, A. J.: Thymosin treatment of children with primary immunodeficiency disease. Transplant. Proc. *10*, 203 (1978)
20. Goldmann, S. F., Niethammer, D., Flad, H.-D., Belohradsky, J., Colombani, J., Dieterle, U., Dosch, H. M., Gelfand, E. W., Töllner, U., Fliedner, Th. M., Kleihauer, E.: Hemopoietic and lymphopoietic split chimerism in severe combined immunodeficiency disease (SCID) Transplant. Proc. *11*, 225 (1979)
21. Teller, W. (Ed.): Rearing of nonidentical twins with lymphopenic hypogammaglobulinemia under gnotobiotic conditions. Acta Paediatr. Scand. Suppl. *240*, 13 (1973)
22. Mittal, K. K., Mickey, M. R., Singal, D. P., Teresaki, P. I.: Serotyping for homotransplantation XVIII. Refinement of microdroplet lymphocyte cytotoxicity test. Transplantation *6*, 913 (1968)
23. van Rood, J. J., van Leeuwen, A., Ploem, J. S.: A method to detect simultaneously two cell populations by two color fluorescence. Its application for the recognition of B-cell (Ia-like) determinants. Nature *262*, 795 (1976)
24. Bodmer, J. G., Pickbourne, P., Richards, S.: Ia-serology. In: Bodmer, W. S. (Ed.): Histocompatibility Test, p. 35. Copenhagen: Munksgaard 1977
25. Goldmann, S. F.: Selection of related and unrelated bone marrow donors. Transplant. Proc. *9*, 507 (1977)
26. Winchester, R. J., Fu, S. M.: Lymphocyte surface menbranes immunoglobulin. Scand. J. Immunol. 5. Suppl. *5*, 77 (1976)
27. Wernet, P., Betsch, C., Barth, P., Jaramillo, S., Schunter, F., Waller, H. D.: Human Ia alloantigens as cell-differentiation markers of normal and pathologic leukocyte surfaces. Scand. J. Immunol. *6*, 563 (1977)
28. Ross, G. D., Polley, M. J.: Assay for the two different types of lymphocyte complement receptors. Scand. J. Immunol. *5*, Suppl. 5, 99 (1976)
29. Niethammer, D., Bienzle, U., Rodt, H., Goldmann, S. F., Körbling, M., Flad, H.-D., Netzel, B., Haas, R. J., Fischer, K., Fliedner, Th. M., Thierfelder, S., Kleihauer, E.: Rhesusincompatibility

and aplastic anemia as the consequence of split chimerism after bone marrow transplantation for severe combined immunodeficiency. Submitted for publication.
30. Niethammer, D., Goldmann, S. F., Flad, H.-D., Bienzle, U., Dieterle, U., Haas, R. J., Heymer, B., Meigel, W., Belohradsky, B. H., Kleihauer, E.: Reconstitution with histoincompatible maternal marrow in a case of severe combined immunodeficiency with graft-versus-host-reaction following materno-fetal transfusion. Submitted for publication.
31. Katz, D. H., Hamaoka, T., Benacerraf, B.: Cell interaction between histoincompatible T- and B-lymphocytes. II. Followup of physiologic cooperative interaction between T- and B-lymphocytes from allogeneic donor strains in humoral response to hapto-protein conjugates. J. Exp. Med. *2,* 1405 (1973)
32. Hoffmann, T., Wang, C. Y., Winchester, R. J., Ferrarini, M., Kunkel, H. G.: Human lymphocytes bearing "Ia-like" Antigens: J. Immunol. *119,* 1520 (1977)

Discussion

Gordon-Smith: What was the relationship between the hemolytic anemia and the aplasia?
Niethammer: The main cause of the hemolytic anemia was very clear: there were rhesus antibodies with anti-D and anti-C antibodies, a real rhesus incompatibility. In addition there were antibodies against all rhesus negative cells. It is most likeley that the aplastic anemia was caused by some kind of immunological mechanism but I dont believe it is the same mechanism that caused hemolytic anemia.
Vriesendorp: There are animal data to suggest that these reactions are caused by GvH reactions. There is a hemolytic anemia in rabbits and there are extensive studies on hemolytic anemia on chronic GvH in mice. Parent-to-F_1 studies have shown that aplastic anemia is one of the consequences of GvH reactions. I do think that both phenomena can be explained on the basis of GvH.

Transplantation of Lymphoid Cells in Patients with Severe Combined Immunodeficiency (SCID)

C. Griscelli, A. Durandy, J. L. Virelizier, and D. Buriot

A. Introduction

There have been successful instances of reconstitution of patients with severe combined immunodeficiencies (SCID) after transplantation of bone marrow, fetal tissue, and, more recently, cultured thymic epithelium [1, 2, 3, 12]. This report summarizes 15 attempts made in our group to correct various types of SCID by grafts of fetal liver and/or thymic cells, or by transplantation of phenotypically or genotypically identical bone marrow tissue. Special emphasis will be given to the complete immunological reconstitution after compatible bone marrow cell transplantation, of SCID patients showing a normal number of B lymphocytes before transplantation. These patients, who appear to have a defect of precursor T cells, were reconstituted by donor marrow stem cells that were able to mature into the host thymus environment, and to become capable of expressing cell-mediated functions and of cooperating with recipient B cells in antibody production.

B. Immunological Evaluation of Patients with SCID

Distribution of patients according to sex was 11 males and 4 females. In four patients, SCID was associated with adenosine desaminase (ADA) deficiency. Among the 11 ADA-positive patients, the family history strongly suggested that two had an autosomic form and five a sex-linked form of the disease. In four other patients, a sex-linked transmission was probable. At the time of their transplant, all patients were less than 8 months old.

The white blood cell counts in the 15 patients showed lymphopenia in six, including the ADA-negative patients, and near normal lymphocyte counts in nine. B-lymphocyte studies were done on 14 patients. Eight of these patients showed an increased number of circulating B cells, but in all 15 cases, the immunoglobulin levels were markedly depressed (except for the presence of maternal IgG). In all 15 cases, the anti-A and anti-B isoagglutinins were undetectable, and no significant rise in antibody titers was obtained following immunization. Data on erythrocyte-rosette-forming cell (E-RFC) number were available for 14 SCID patients. In all cases, the E-RFC were less than 1 percent. Skin tests were negative. Mitogenic responses were evaluated after stimulation with various polyclonal mitogens or allogeneic cells. The response to phytohemagglutinin (PHA), concanavalin A (Con A), or pokeweed mitogen (PWM) was undetectable. In four patients showing an increased number of B cells, there was

a significant in vitro response after allogeneic stimulation. These allogeneic responses (MLR) were shown to be due to a proliferation of patient B cells with the help of mitomycin-treated or of irradiated leukocytes from normal donors. Stimulation of patient leukocytes with either mitomycin-treated Raji cells (a pure B cell suspension) or purified normal adherent monocyte did not induce any detectable proliferation, suggesting that patient B cells were capable to respond in MLR only with the help of normal T lymphocytes. The leucocytes from three patients could not stimulate normal allogeneic cells, although HLA-A and B antigens were normally detected on their membrane. Serology of the HLA-D membrane antigen was not performed. Leucocytes from two SCID patients with ADA deficiency showed a normal response and stimulation ability in the MLR assay. Furthermore, cytotoxic cells were generated during MLR in these two patients.

C. Grafting of Fetal Tissue

One patient received fetal thymus alone and four other patients received fetal thymus and liver cells (i.v. or i.p.). The fetuses were obtained from neighbouring hospitals in Paris and varied in age from 13 to 20 weeks of gestation. In each case, cell suspensions were prepared and injected within 2hr following histerotomy. Cell viability was more than 90 percent by trypan blue exclusion. The characteristics of these grafts are summarized in Table 1. Patient Y. D., A. J. and R. P. died from infections within 15 days to 3 months after the graft. In two cases, partial T and B-cell reconstitution was observed. A rapid graft versus host disease (GVHD) occured in patient 4 indicating that fetal tissues can induce a fatal GVHD. In one SCID patient with ADA deficiency, three successive attempts of reconstitution with fetal tissues were unsuccessful. It seems probable that rejection of fetal tissues occured in this peculiar patient with normal MLR and generation of cytotoxic cells. Altogether, our results with these patients are quite desappointing since 1-GVHD can occur and be lethal 2- only a partial and transitory reconstitution can be obtained and 3- a rejection of the graft can be

Table 1. Results of fetal tissue transplantation (FTT) in SCID (Groupe des Enfants Malades Paris, Jan. 1979)

Patient[a]	Sex	Fetal tissue[b]	Number of cells (10^7/kg)	GvH	Reconstitution		Post-graft survival (months)	Follow-up
					T	B		
1 (1) A. S. M		TH (20)	2.5	±	++	0	11	Pneumocytosis
2 (7) Y. D. M		TH+L (13)	NA[c]	0	+	+	2.9	Septicemia
3 (5) A. J. M		TH+L (14)	NA[c]	0	0	0	0.5	Septicemia
4 (3) R. P. M		TH+L (14)	2.5+80	+++	±	±	2	Septicemia
5 (3,7,9) J. S. F		TH+L (12–14)	3+60[d]	−	−	−	13	+GVHD

[a] Numbers in parentheses represent age (months) at the time of FTT
[b] TH, Thymus: L, Liver: numbers in parentheses represent fetal age (weeks)
[c] NA, not available
[d] Third transplantation

observed when residual T-cell activity is present, particularly in ADA deficiency. A complete restoration of cell mediated immunity was obtained in patient 1, which contrasted with a complete failure humoral reconstitution during the 11 months of survival. His death, due to a pneumocystosis, occured after progressive decrease in the number and functions of the grafted T-lymphocyte. Infection was also the cause of death in the three other cases.

D. Grafting of Bone Marrow Tissue

Ten patients received bone marrow cell transplantation. The results are summarized in Table 2. Six bone marrow transplants were HLA-A, -B, -D-genoidentical. The other four were HLA-A, -B-phenoidentical. Mixed leukocyte reactions were negative (proliferative index less than 2) in all cases, except patient L.T. in whom it was difficult to assess the response to allogeneic cells because of a high spontaneous incorporation of labeled thymidine. The appearance of severe GVHD in this case suggested that the donor and the recipient were not identical at the D locus. In six other patients who survived long enough, mild signs of GVHD were observed, characterized by transitory (15 days) or persisting (several months) eosinophilia and skin rash, but no or mild gastrointestinal involvement. Definite signs of T and B cell reconstitutions were obtained in eight cases, and chimerism was demonstrated in two of them by

Table 2. Results of bone marrow transplantation (BMT) in SCID (Groupe des Enfants Malades, Paris, Jan. 1979)

Patient[a]	sex	Donor	Typing compatibility HLA			Number of cells (10^7/kg)		Reconstitution		Postgraft survival (months)	Follow up
			ABO	AB	MLR		GVHD	T	B		
1 (8) O. B.	M	Father	+	+	+	92	?	0	0	0.3	Pneumo catosis
2 (7) Y. R.	M	Sister	+	+	+	35	?	?	?	0.1	Pneumo-cytosis
3 (9) Y. O.	M	Sister	+	+	+	100	±	+++	+++	2	Sequelae of meningitis
4 (3) L. N.	M	Grand Mother	+	+	+	6.5	+	++	+	2	Graft versus graft reaction
5 (3) [c]L. T.	M	Grand Aunt	+	+	?	30	++	++	±	2	GVHD
6 (2) S. A.	F	Brother	+	+	+	49	±	+++	+++	79[b]	Healthy
7 (2) [c]M. B.	M	Brother	+	+	+	27	+	+++	++	42[b]	Healthy
8 (4) C. L.	F	Sister	+	+	+	40	±	+++	+++	31[b]	Healthy
9 (1.5) G. N.	M	Aunt	+	+	+	30	+	+++	++	25[b]	Healthy
10 (3) [c]J. T.	F	Sister	+	+	+	45	±	+++	+++	8	Healthy

[a] Numbers in parentheses represent age (months) at the time of BMT
[b] Proven chimerism
[c] ADA negative

karyotyping, Y chromosome staining with quinacrine [4] or HLA typing. In patients 7 and 10, the normal ADA activity found in circulating lymphocytes suggested that these cells were of donor origin. Patient 8 (female) was reconstituted with marrow cells from an HLA-identical sister and showed red blood cells markers of donor origin.

Two patients died before any sign of take (patients O. B. and Y. R.). Three other patients died despite evidence of reconstitution. In patient Y. D, death was caused by the neurological sequelae of various episodes of meningitis experienced before grafting. Upon post mortem examination, B and T cell compartments could be visualized in most lymphoid organs, except the lymph nodes and Peyer patches [6]. Patient L. N. was grafted with cells from an HLA-A, B, -D-identical grandmother. Before grafting, he had been transfused twice with irradiated packed blood cells from his father. Although these cells had been irradiated with 5000 rads, they were present two months later, by the time of the marrow transplant and could be detected by HLA typing and radiation-induced chromosomal abnormalities. This special situation resulted in a fatal graft-versus-graft-reaction. In the five surviving patients, complete and persisting reconstitution of humoral and cell-mediated immune functions was obtained. These patients are doing well, and remain free of infections.

E. Immunological Dysregulation Associated with Mild GVHD

Even under conditions of complete HLA: A, B, D identity, some GVHD reaction was observed after bone marrow transplantation. This produces a complex immunological situation, with alternative phases of correction and disturbance of various immunological parameters [18]. In patient M. B., for instance, fifteen days after transplantation, the number of circulating lymphocytes increased abruptly and this was followed shortly by an intense eosinophilia, which peaked at about 30,000/mm^3 on day 40 after transplantation. A characteristic skin rash was also observed at that time, further suggesting the development of a GVHD reaction. This period was characterized by the appearance of T cell markers in the peripheral blood, with a concomitant rise in lymphocyte reactivity to mitogens. The IgM titer in the serum rose to 170 mg%, an exaggerated level at that age. However, after this period of intense immunological activity, a progressive decrease in lymphocyte counts and E-RFC and serum IgM levels was observed. Three months after transplantation, the three later immunological markers had returned to levels comparable to those observed before transplantation. In contrast, at that stage, proliferations induced by PHA and Con A was very intense. This, together with the finding of a subnormal level of ADA activity in the patient's peripheral lymphocytes, indicated that immunological reconstitution had taken place and persisted. It was thus decided not to perform any new bone marrow transplantation. In an attempt to stimulate the immunological system, five injections of Corynebacterium parvum were administered intramuscularly. This was followed shortly by another rise in the number of peripheral lymphocytes and eosinophils, with a concomitant rise in E-RFC and in serum IgM. In contrast, in vitro reactivity to mitogens diminished to almost undetectable

levels. This second period of intense immunological activity was again followed by a progressive decrease in E-RFC and in serum IgM levels. A stabilization of immunological markers was eventually obtained, and the patient was successfully taken out of the sterile environment. He is now fully reconstituted, 42 months after bone marrow transplantation. The study of patients S. A., C. L. and J. T. showed similar variations in immunological parameters during the months following transplantation. Thus, signs of immunological dysregulation appear to be frequent after such transplantation.

F. Defect of Precursor T Cells in SCID with B Lymphocytes

Recently, SCID patients have been described with peripheral B lymphocytes in normal number but unable to mature into antibody-producing cells [5, 11, 17]. Eight such patients are described in the present series. In two cases (patients S. A. and G. N.), a phenotypically or genotypically identical marrow transplantation allowed the development of a thymus that became visible on X-ray examination, strongly suggesting that the primary defect was a selective abnormality of bone marrow precursor T cells, at a prethymic level.

Almost all circulating lymphocytes (95 to 100 percent) in these two patients carried surface Ig determinants, with a large percentage bearing both μ and δ chains. These cells also bore receptors for complement and were stained by a fluorescent anti serum specific for B cells. In vitro studies of the maturation of the cells of several patients (A. S., R. P., G. N.) in the presence of PWM and normal T cells suggested that the B cells remained blocked at the level of μ and δ chain expression, perhaps due to an absence of T cell helper function. Indeed, in the presence of PWM and normal T cells, the B cells of these patients matured into Ig containing cells. Following grafting, patients (S. A. and G. N.) were shown to carry T-cells of donor origin by karyotyping or fluorescent staining of Y chromosome with quinacrine. Lymphocytes with surface or intracytoplasmic immunoglobulins were from the recipients. Allotype studies by Dr. L. Rivat of anti-A isohemagglutinins present in the serum of patient G. N. after repeated immunization with blood group substances revealed the presence of a Gm factor not present on the immunoglobulins of the donor. Taken together, these studies demonstrated that host B-cells were able to cooperate with donor T cells in immunoglobulin and antibody production. Why the donor's B cells, presumably present in the marrow graft, were never identified in the recipient is a matter of speculation. The peripheral monocytes and granulocytes also carried the recipient's markers after reconstitution, only the missing lineage being readily replaced.

G. Conclusion

Our studies indicate that, even in the group of patients with B-cells, bone marrow transplantation is the best way to reconstitute cellular and humoral functions in patients with SCID. The slow maturation in host thymus of T cells capable of

cooperation with the recipient's B cells may take several months. In several cases, the take was accompanied by a mild GVHD, associated with alternative increases and decreases of various immune functions which lasted for several months. The benefits of bone marrow transplantation were awaited by keeping the patients in a Trexler isolator until immune competence was obtained. This made is possible to avoid reinjection of new marrow cells. Unfortunately, a histocompatible bone marrow donor is not always available. In patients with a prethymic deficiency, the logical second best strategy seems to be the injection of precursor cells from a fetus. In fact, several fetal liver or thymus grafts have been used to reconstitute partially SCID patients [1, 3, 14, 16]. A thymus graft can, on one hand, correct epithelial function to allow the maturation of recipient stem cells. On the other hand, thymocytes present in the graft can directly reconstitute the T-cell compartment, with the risk of GVHD. The recent report stating that various cases of SCID have benefited from injections of cultured epithelium from normal thymus offers a new therapeutic approach that does not entail the risk of GVHD [12]. It remains to be seen whether this procedure will be effective in patients who do not seem to have an intrathymic defect.

Severe combined immunodeficiency is an heterogeneous disease and every effort should be made to assess the level of the defect. Preliminary studies indicate that in vitro culture of patient's stem cells in the presence of normal thymus epithelium [15] or determination of serum thymic factor [13] may be helpful in this respect.

H. Summary

The effects of bone marrow or fetal lymphoid organ transplants in 15 patients with severe combined immunodeficiency disease are reported. The benefits and dangers inherent to the various transplantation strategies are discussed. Our studies indicate that, even in the group of patients with B cells, bone marrow transplantation may be the best procedure to obtain the reconstitution of cellular and humoral functions.

Acknowledgements

This work was supported by INSERM grants (ATP 7.74.28 and 8.74.29) and Fondation pour la Recherche Médicale Française.

References

1. Amman, A. J., Wara, D. W., Salmon, S., Perkins, H.: Thymus transplantation. Permanent reconstitution of cellular immunity in a patient with sex-linked combined immunodeficiency. N. Eng. J. Med. *5*, 5 (1973)
2. Buckley, R. H.: Reconstitution: grafting of bone marrow and thymus. In: Amo D. B. (Ed.): Progress in Immunology, p. 1061. New York: Academic Press 1971

3. Buckley, R. H., Whismant, J. K., Schiff, R. I., Gilbersten, R. B., Huang, A. T., Platt, M. S.: Correction of severe combined immunodeficiency by fetal liver cells. N. Eng. J. Med. *294,* 1076 (1976)
4. Capersson, T., Zech, L., Johansson, C., Modest, E. J.: Identification of human chromosomes by DNA binding fluorescent agents. Chromosoma *30,* 215 (1970)
5. De Fazio, S., Criswell, B. S., Kimzey, S. L., South, M. A., Montgomery, J. R.: A paraprotein in severe combined immunodeficiency disease detected by immunoelectrophoresis analysis of plasma. Clin. Exp. Immunol. *19,* 563 (1975)
6. Dooren, L. J., Kamphuis, R. P., De Koning, J., Vossen, J. M.: Bone marrow transplantation in children. Semin. Hematol. *11,* 369 (1974)
7. Geha, R. S., Schneeberger, E., Gatien, J., Rosen, F. S.: Synthesis of an M-component by circulating B lymphocytes in severe combined immunodeficiency. N. Eng. J. Med. *290,* 726 (1974)
8. Good, R. A., Bach, F. H.: Bone marrow and thymus transplants: Cellular engineering to correct primary immunodeficiency. In: Bach, F. H., Good, R. A. (Eds.): Clinical Immunobiology, Vol. 2, p. 63. New York: Academic Press 1974
9. Griscelli, C.: T and B markers in immunodeficiencies. In: Bergsma, D. (Ed.): Birth Defects: Immunodeficiency in Man and Animals, Vol. 11, p. 45. New York: The National Foundation March of Dimes 1975
10. Griscelli, C.: Bone marrow transplantation in SCID. In: Bergsma, E. (Ed.): Birth Defects: Immunodeficiency in Man and Animals, Vol. 11, p. 426. New York: The National Foundation March of Dimes 1975
11. Griscelli, C., Durandy, A., Virelizier, J. L., Ballet, J. J., Daguillard, F.: Selective defect of precursor T cells in severe combined immunodeficiency with B lymphocytes. J. Pediatr. *93,* 404–411 (1978)
12. Hong, R., Santosham, M., Schulte-Wissermann, H., Horowitz, S., Hsu, S. F., Winkelstein, J. A.: Reconstitution of B and T lymphocyte functions in severe combined immunodeficiency disease after transplantation with thymic epithelium. Lancet *II,* 1270 (1976)
13. Incefy, G. S., Dardenne, M., Pahwa, S., Grimes, E., Pahwa, R., Smithwick, E., O'reilly, R., Good, R. A.: Thymic activity in severe combined immunodeficiency disease. Proc. Natl. Acad. Sci. USA Vol. *74,* 1250–1253, March 7, Medical Sciences (1978)
14. Keightley, R. G., Lawton, A. R., Cooper, M. D., Ynis, E. J.: Successful fetal liver transplantation in a child with severe combined immunodeficiency. Lancet *II,* 850 (1975)
15. Pyke, K. W., Dosch, H. M., Ipp, M. M., Gelfand, E. W.: Demonstration of an intrathymic defect in a case of severe combined immunodeficiency disease. N. Eng. J. Med. *293,* 424 (1975)
16. Rachelefsky, G. S., Stiehm, E. R., Amman, A. J., Cederbaum, S. D., Opelz, G., Terasaki, P. I.: T cell reconstitution by thymus transplantation and transfer fact in severe combined immunodeficiency. Pediatr. *55,* 114 (1975)
17. Seeger, R. C., Robins, R. A., Stevens, R. H., Klein, R. B., Waldman, D. J., Zeltzer, P. M., Kessler, S. W.: Severe combined immunodeficiency with B lymphocytes: In vitro correction of defective Ig production by addition of normal T lymphocytes. Clin. Exp. Immunol. *26,* 1 (1976)
18. Virelizier, J. L., Durandy, A., Ballet, J. J., Griscelli, C.: Immunological dysregulation after bone marrow transplantation in patients with severe combined immunodeficiency. Pathol. Biol. (Paris) *26,* 21–22 (1978)

Y-Chromatin: Method to Study a Take or Rejection After Bone Marrow Transplantation*

Renate Arnold, H. Heimpel, K.-P. Hellriegel, H. Pflieger and U. Vetter

To study a take or rejection after bone marrow transplantation, chromosome analysis is routinely done when donor and recipient are of different sex or another marker chromosome is present.

Caspersson et al. [1, 2] showed that quinacrine mustard stains defined parts of chromosomes by effecting characteristic patterns of fluorescent bands thereby improving the identification of chromosomes. Quinacrine dihydrochlorid selectively stains the distal part of the long arms of the human Y-chromosome, which is demonstrable in metaphase as well as in interphase nucleus of a leukocyte [8, 9, 10, 11]. In interphase nucleus the Y-chromosome appears as strongly fluorescent body called Y-chromatin.

In order to study a take or rejection of the graft we studied the presence of the Y-chromatin in interphase nuclei of leukocytes from the peripheral blood of female patients who had received bone marrow transplantation from male donors.

A. Material and Methods

3 female patients with aplastic anemia transplanted with bone marrow from their HLA-identical, MLC-negative brothers were studied.

For identification of the Y-chromatin, peripheral blood smears were fixed with 98% methanol and stored at 4° C until staining. The slides were stained with 0.5% aqueous quinacrine dihydrochloride solution for 10 minutes. The smears were then washed for 2 minutes under tap water and covered with a cover slip. A Zeiss universal microscope equipped with HBO 200 W/4 light source and a BG 3,12,436 emission filter in combination with a 50/40 transmission filter, was used in vertical illumination fluorescence for evaluation [6]. Each time 100 polymorphonuclear neutrophiles (PMN) and 100 lymphocytes were evaluated. As control leukocytes of healthy men and women were studied. The Y-chromatin positive cells ranged from 80–90% in men and from 0–5% in women.

Cytogenetic analysis of aspirated bone marrow cells was done in direct marrow preparation [7].

B. Results

Patient D. B. (Fig. 1): The patient was transplanted from her HLA-identical, MLC-negative brother. She received 3.65×10^8 bone marrow cells/kg body weight. From day 1 to day 16 after bone marrow transplantation the patient received prophylactic granulocyte transfusions from the bone marrow donor.

* Supported by Deutsche Forschungsgemeinschaft, SFB 112

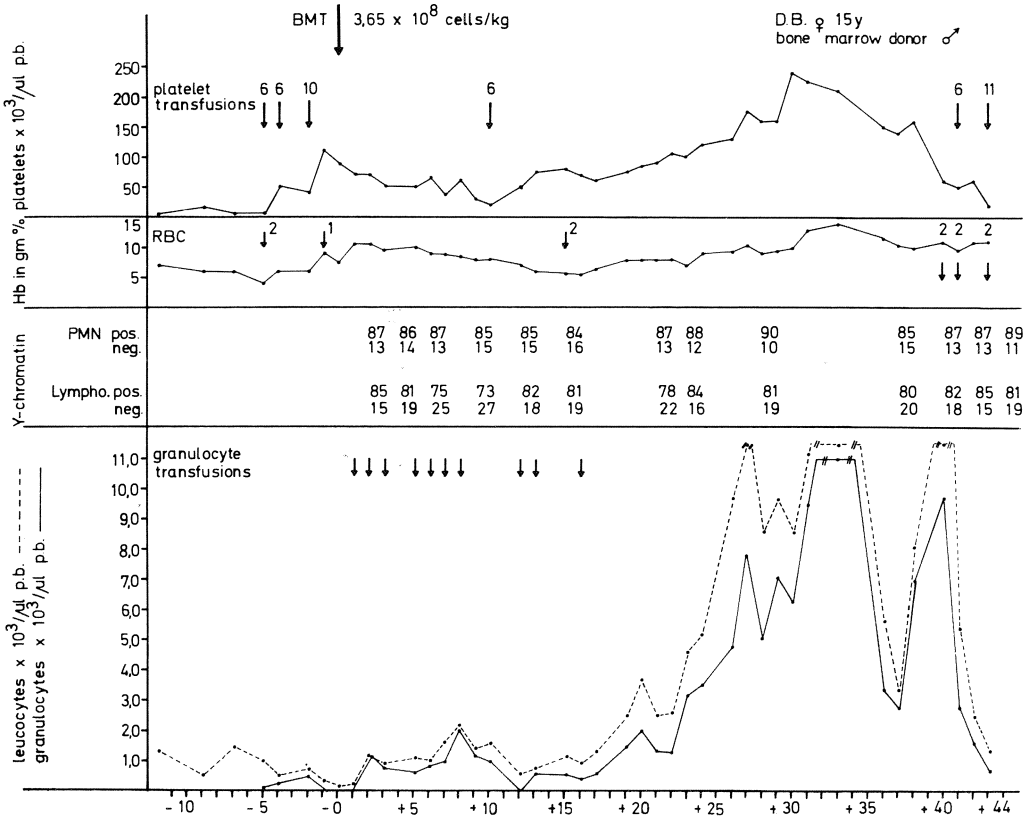


Fig. 1. Patient D. B.

After the third week the patient developed GVHD and died on day 44 because of severe GVHD and septicemia.

During granulocyte transfusion therapy from the bone marrow donor from day 2 onwards, 80–90% of PMN's as well as lymphocytes of the peripheral blood showed the presence of the Y-chromatin, indicating that the leukocytes were of male origin. After granulocyte transfusions were stopped 80–90% of the peripheral blood PMN's as well as lymphocytes showed the presence of Y-chromatin indicating the presence of male type cells. This could be shown during the follow up period from day 21 to day 44. As control, cytogenetic analysis from aspirated bone marrow cells were done at weekly intervals. Only metaphases of male type were found, thus confirming a take after BMT.

Patient F. H. (Fig. 2): The patient was transplanted from her HLA-identical, MLC-negative brother. She received $1{,}28 \times 10^8$ bone marrow cells per kg body weight. On day 4 granulocyte transfusions were started for treatment of septicemia. The patient died on day 16 because of DIC and gramnegative septicemia. On day 4, 94% of PMN's of peripheral blood showed the presence of the Y-chromatin, indicating that the PMN's were of male origin. After granulocytes from the mother were given, only 13% of PMN's of the peripheral

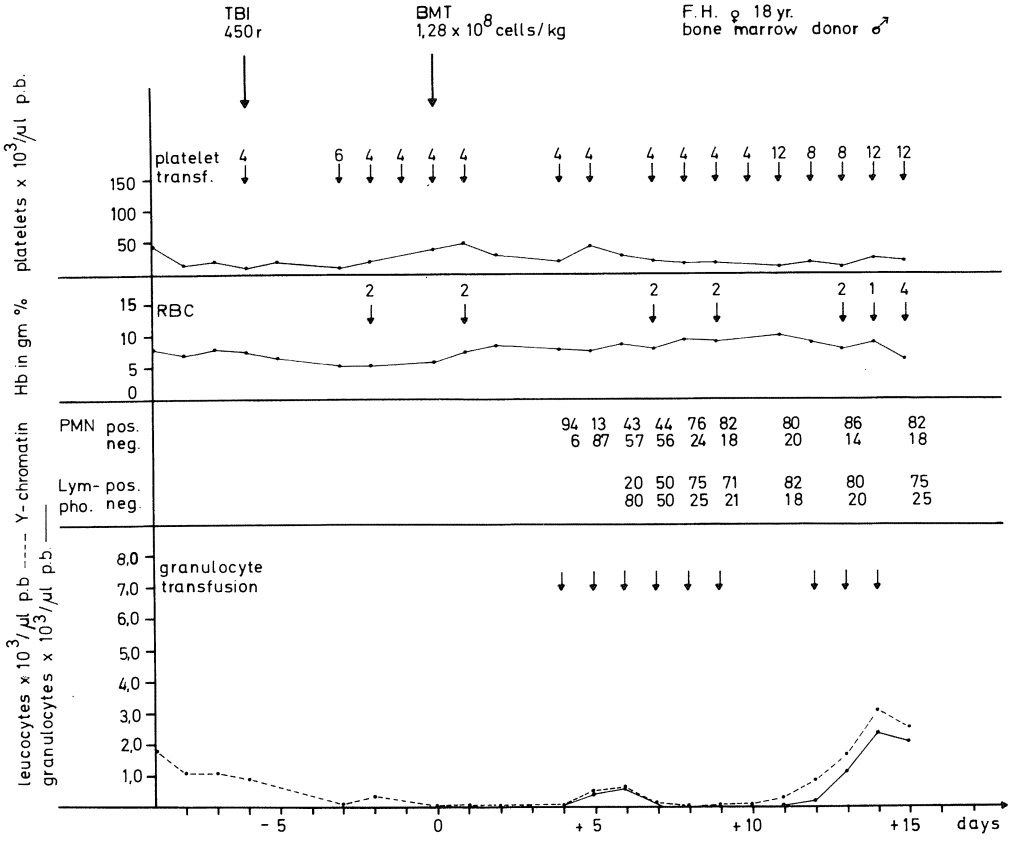


Fig. 2. Patient F. H.

blood showed the presence of the Y-chromatin, meaning most of the PMN's were of female origin. After granulocytes of the mother and an unrelated male donor were given, on day 6 and 7, 43% and 44% respectively of the PMN's and 20% and 50% respectively of the leukocytes showed the presence of the Y-chromatin. During granulocyte transfusions from male donors from day 8 onwards, 76, 82 and 86% respectively of PMN's and 75, 71 and 80% respectively of lymphocytes of the peripheral blood showed the presence of Y-chromatin. On day 11 and day 16, when no granulocytes were given, 80% and 82% respectively of PMN's and 82% and 75% respectively of lymphocytes showed the presence of the Y-chromatin, indicating that the leukocytes were of male origin, that is bone marrow donor type.

Our finding of a take using the presence of Y-chromatin was confirmed by bone marrow aspirates on day 7 and post mortem showing regeneration of the bone marrow.

Patient F. F. (Fig. 3): The patient was transplanted from her HLA-identical, MLC-negative brother. She received $2{,}20 \times 10^8$ bone marrow cells per kg body weight. Bone marrow aspirates on day 14, 21 and 28 after BMT showed increasing bone marrow cellularity. On day 41 rejection of the graft was

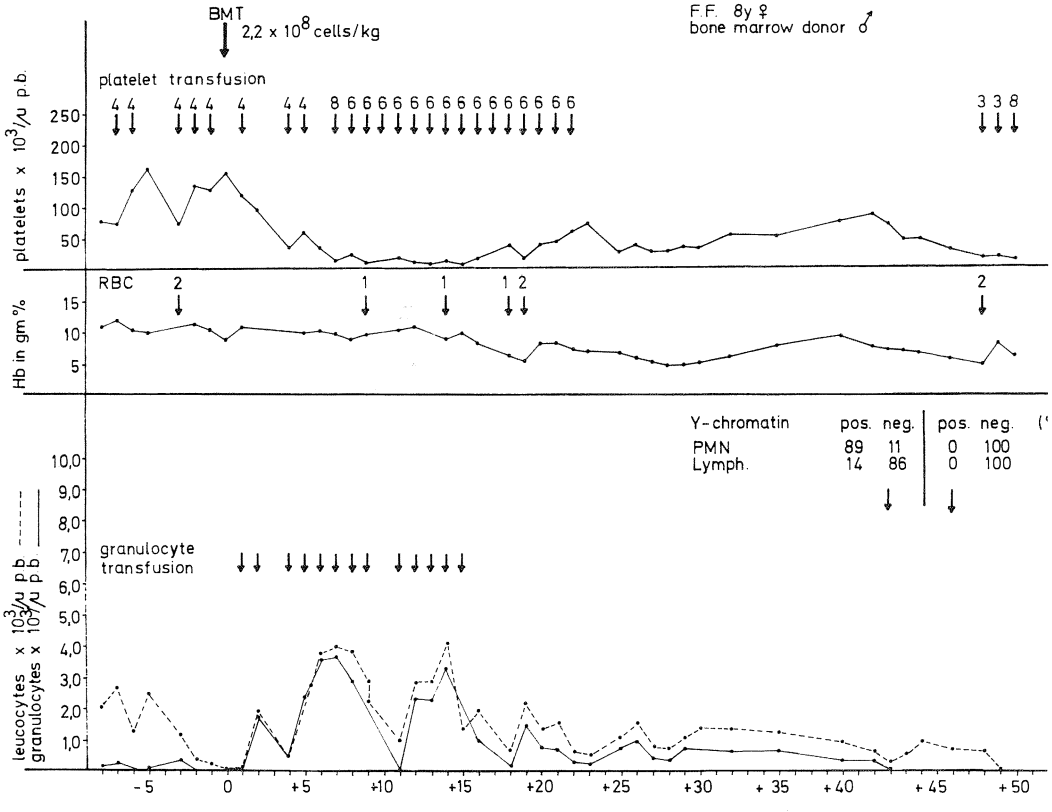


Fig. 3. Patient F. F.

suspected when fever of unknown origin together with decrease of peripheral PMN's and reticulocytes occured.

On day 42, 89% of the PMN's of the peripheral blood showed the presence of the Y-chromatin, indicating that the PMN's were of male origin, that is donor origin, 14% of the lymphocytes of the peripheral blood showed the presence of the Y-chromatin, indicating that most of the lymphocytes were of female origin that is recipient origin. In the chromosome analysis 44 metaphases were of female type, that is cells of recipient origin, 10 metaphases were of male type, that is cells of donor origin.

On day 46, 0% of the PMN's as well as lymphocytes of the peripheral blood showed the presence of the Y-chromatin, indicating that the leukocytes were of female origin, that is recipient origin.

C. Discussion

Presence or absence of the Y-chromosome in blood or bone marrow cells is still the most reliable marker to prove or disprove take of the donor hemopoietic cells in allogeneic bone marrow transplantation between donor and recipient of

opposite sex. It may be useful in predicting a graft rejection after primary take by the reappearance of recipient type lymphocytes. It may also be useful in identifying the rare occurence of leukemia in the blood cells of donor origin [4, 5]. As shown by Caspersson and associates [1, 2], identification of the Y-chromosomes may be facilitated or replaced by specific fluorescence of the Y-chromosome after atebrin or acridine staining. The use of this method to study blood and bone marrow cells after bone marrow transplantation will offer the following advantages:

1. Non dividing cell populations (no mitosis) may be analysed, because Y-fluorescence is also present in interphase nuclei.
2. The technique is less time consuming and results can be obtained within a short period of time.
3. The morphological characteristics of the cell, i.e. lymphocytes, PMN's, is easily recognized.

Despite these advantages, there are only few reports of the use of the Y-fluorescence technique in human bone marrow transplantation [4, 5, 12]. We studied interphase nuclei of leukocytes in the peripheral blood of 3 female patients with aplastic anemia transplanted with bone marrow from HLA-identical brothers. In case 1, all peripheral nucleated cells obtained after BMT were of male origin, both during the period of intensive granulocyte substitution from the donor as well as during the period of repopulation by the transplanted hemopoietic cells. These results were confirmed by analysis of metaphases from bone marrow cells. In case 2, a take was demonstrated by Y-fluorescence, but female cells appeared for a short time after granulocyte substitution from the mother. In case 3 male (donor) characteristics were seen in the PMN's but not in the lymphocytes in the beginning of graft rejection. Later only recipient cells were seen when rejection was complete. Serial Y-fluorescence data are not available from this patient, but the few data indicate the possibility to detect a rejection early by the reappearance of recipient lymphocytic cells.

The quantitative analysis of cell populations is influenced by a possibility of error of the method due to the presence of fluorescence bodies derived from chromosomes other than Y. These are also seen in interphase nuclei and metaphases [3]. However, this "non-Y" background may be kept rather low by suitable preparations and experience of the observer. Usually, the interphase Y-fluorescence body showes stronger fluorescence than the other fluorescence bodies, e.g. interphase 3 and interphase D. Moreover, the Y-fluorescence body is often peripherally located near the nuclear membrane, whereas 3 and D interphase bodies are located closer to the center of the nucleus.

In conclusion, the observations reported show that Y-fluorescence of interphase cells can be a useful technique to study take and rejection in allogeneic human bone marrow transplantation.

References

1. Caspersson, T., Zech, L., Johansson, C.: Exp. Cell. Res. *60,* 315–319 (1970)
2. Caspersson, T., Zech, L., Johannsson, C., Modest, E. J.: Chromosoma *30,* 215–227 (1970)
3. Caspersson, T., Zech, L., Johansson, C., Lindsten, J., Hulten, M.: Exp. Cell. Res. *61,* 472–474 (1970)
4. Elfenbein, G. J., Brogaonkar, D. S., Bias, W. B., Burns, W. H., Saral, R., Sensenbrenner, L. L., Tutschka, P. J., Zaczek, B. S., Zander, A. R., Epstein, R. B., Rowley, J. D., Santos, G. W.: Blood *52,* 627–636 (1978)
5. Fialkow, P. J., Thomas, E. D., Bryant, J. J., Neiman, P. E.: Lancet *I.,* 251–255 (1971)
6. Hellriegel, K. P., Borberg, H., Reitz, H., Gross, R.: Transplant. Proc. *4,* 399–403 (1974)
7. Lam-Po-Tang, P. R. L. C.: Scand. J. Haematol. *5,* 158–160 (1968)
8. Majewski, F., Bier, L., Pfeiffer, R. A.: Klin. Wochenschr. *49,* 814–818 (1971)
9. Pearson, P. L., Bobrow, M., Vosa, C. G.: Nature *226,* 78–80 (1970)
10. Polani, P. E., Mutton, D. E.: Br. Med. J. *1,* 138–142 (1971)
11. Schröder, J., De La Chapelle, A.: Blood *39,* 153–162 (1972)
12. Storb, R., Thomas, E. D., Weiden, P. L., Buckner, C. D., Clift, R. A., Fefer, A., Fernando, L. P., Giblett, E. R., Goodell, B. W., Johnson, F. L., Lerner, K. G., Neiman, P. E., Sanders, J. E.: Blood *48,* 817–841 (1976)

Discussion

Tutschka: We have been using the Y chromatin study for other cells for instance on cryosections.
Arnold: This was first described by Don Thomas in a study of macrophages of the lung.
Santos: It is also very useful in the recurrence of tumors, for instance in a patient with chloroma.
Kersey: I would like to add one additional use of your method in a patient with osteopetrosis, an abnormality in the formation of osteoclasts which was transplanted with allogeneic bone marrow, a male into a female. The disease improved dramatically and we were able to demonstrate male osteoclasts.

9 Summing up

Summing up in Perspective

D. W. van Bekkum

The papers presented during this meeting cover a diversity of subjects directly or indirectly related to bone marrow transplantation, which may be grouped into three main categories: basic and applied experimental research and clinical experience. In the following discussion, certain recent developments not specifically reported at the conference will be taken into account, as some of them came up during the discussions and because others can simply not be ignored in an up to date review of this kind.

A. Basic Research

The fundamental aspects of bone marrow transplantation are obviously part of experimental hematology. Here the main interests for bone marrow transplantation are:
1. The pluripotent hemopoietic stem cell (HSC) which is thought to be the essential component of bone marrow that has to take, and
2. the mechanisms which govern the development of immune competent T cells which are thought to be the culprits in the initiation and maintenance of graft-versus-host disease (GVHD).

I. Microenvironment

The hemopoietic microenvironment is presently being studied in culture systems following the development by Dexter of a method which allows a regular multiplication of CFU-s over a period of several months. Dexter et al. [5] have suggested that the feeder layer which is required in the cellular system might reflect by its complexity the in vivo situation. They described effective feeders to consist of 3 cell types: macrophages (phagocytic mononuclear cells), giant fat cells (very large cells containing fat) and flattened epithelial-like cells. In contrast, Reimann and Burger [14] in the Radiobiological Institute TNO have collected evidence in favour of only one cell type, a fibroblast, being required for CFU-s multiplication. They showed that in successive passages of bone marrow fibroblasts the proportion of macrophages decreases drastically, yet feeder layers depleted of macrophages allow CFU-s multiplication just as well as the primary bone marrow cultures. Giant fat cells appeared in these feeder layers only when the feeder aged and this was again not a requirement for CFU-s multiplication. They observed as others did, that the bone marrow contains fibroblast colony forming cells and this will permit to put their hypothesis to the crucial test by

investigating the potential of a clone derived feeder layer to maintain CFU-s multiplication. The assumed exclusive role of fibroblasts in supporting CFU-s replication is in accordance with earlier observations of Dicke et al. who demonstrated a slight but significant production of CFU-s in mouse bone marrow colonies in a soft agar system provided with a feeder layer of embryonal fibroblasts [7].

The microenvironment of the thymus is required for the timely reconstitution of the T lymphocyte (T ly) compartment in radiation chimeras. In thymectomized recipients of isogeneic bone marrow, T cell function remains absent or minimal for a long time. It is less generally known that eventually – after as long as 6–12 months – partial recovery does take place [1]. The recent observations of Zinkernagel et al. [25] have shown that certain T cell functions such as the cytotoxic response to virus infected target cells and T cell helper function are restricted by the H2 type of the thymus. According to this restriction, allogeneic A → B bone marrow chimeras cannot develop T cell responses because in these chimeras the T cells are B restricted (the origin of the thymus) while antigen is presented by A cells. Evidence was provided in this conference that the thymus-directed H2 restriction is not a general phenomenon applicable to other situations than those studied by Zinkernagel. Allogeneic thymus does enable T cell precursors to develop helper function in nude mice (Kindred, this symp.) and in radiation chimeras (Rodt et al., this symp.) as well as secondary CTL reactions in radiation chimeras (Wagner et al., this symp.). These new findings do not uphold the explanation that the defective immune reconstitution shown by allogeneic radiation chimeras is due to thymus H2 restriction. It would rather seem that the defective immune reconstitution as seen in many allogeneic chimeras has to be attributed to persisting clinical or subclinical GVH reactions. This would predict that successful prevention of GVH will also lead to more rapid reconstitution of T cell immunity.

II. Split Chimerism

The proliferative properties of committed stem cells as compared to pluripotent stem cells are becoming an important issue for investigation in view of the increasing interest in split chimerism. Split chimerism leading to partial immunological reconstitution has been described in a number of transplanted SCID (severe combined immune deficiency) patients, but little insight is available into the mechanisms by which this occurs. Niethammer et al. (this symp.) reported on 3 such cases in this conference, all of whom developed various degrees of GVHD since the donor cells were not fully matched. Slavin (this symp.) on the other hand reported the absence of GVHD following transplantation of H2 different bone marrow in mice conditioned by total lymph node (TLN) irradiation, which resulted in mixed chimerism. The induction of stable mixed and split chimerism deserves an increased investigational effort as certain categories of patients, e.g., SCID and aplastic patients, require reconstitution of only one differentiation series of the hemopoietic system. There are some older observations in rodents which suggest that one hemopoietic series can be maintained independently of the others for long periods of time: rat type

granulopoiesis and mouse type erythropoiesis persisted together for as long as a year in some xenogeneic chimeras [24] and erythropoietic reconstitution with some persistance of host cells in lymphoid tissues occurred in W/W^v mice following transplantation of normal bone marrow [16]. It would be desirable to test whether such split chimeras can be obtained by transplanting certain types of committed stem cells in the absence of pluripotent HSC. The differences in size and density of these categories of cells are, however, so small that a separation based on these physical properties does now seem feasible yet. Other properties such as the density of surface antigens, of electrophoretic mobility and of specific dye uptake are not being pursued for the selective isolation of one or more committed stem cell types.

B. Applied Research

I. Conditioning of the Recipient

For many years the conditioning regimens used in the clinic have been limited to total body irradiation (TBR) with approximately 1000 rad gamma radiation delivered at a relatively low dose rate of 5 rad/min, to high dose cyclophosphamide (cyclo) with or without ALG and procarbazide, and to a combination of TBR with cyclo. The latter regimen has been used for leukemics with the primary objective of maximum eradication of leukemic cells. Under this heavy conditioning, take failures were rare to occur even in sensitized patients, but it proved to be too toxic for patients with aplastic anemia. In aplasia, the favoured conditioning with cyclo resulted in a considerable proportion of take failures. Dissatisfaction with the existing conditioning regimens has led to the introduction of modifications which are now being explored in clinical situations with encouraging results. The significance of these developments is that the whole issue of conditioning is once more open for new approaches. Slavin (this symp.) advocates the use of fractionated TLN irradiation according to the regimen employed in the radiotherapy of Hodgkin's disease. This was based on observations by Kaplan's group in thus treated Hodgkin patients, where they found that T cell functions remained severely depressed for 5 years and even longer. This led them to explore the feasibility of TLN irradiation as a conditioning for allogeneic tissue transplantation in mice, rats and dogs, as well as for bone marrow transplantation [20]. As a conditioning period of 3 weeks is not attractive, Kersey et al. (this symp.) introduced a single dose TLN irradiation combined with a course of cyclo and obtained takes of H2 incompatible marrow in mice and of MHC identical marrow in 5/6 pretransfused humans, so far without occurrence of GVHD. The same conditioning followed by MHC mismatched bone marrow grafts in two patients resulted in rejection in one and fatal GVHD in the other.

On the basis of an extensive analysis of experiments in mice, rats, monkeys and dogs, Vriesendorp (this symp.) proposes the use of a lower dose of TBR (1×500 rad) in combination with 2×50 mg of cyclo/kg body weight for conditioning of MHC identical bone marrow grafting. He also advocates the

transplantation of a lower cell number (1.6×10^8 cells/kg body weight) to minimize the risk of GVHD in non-sensitized recipients. The clinical application of this approach opens the way for modifications of the bone marrow graft (i.e., removal of T lymphocytes by density centrifugation or by treatment in vitro with specific antisera) because a certain unavoidable loss of stem cells inherent to these procedures, can now better be tolerated.

A lowering of TBR dose may also be the essential factor in the approach developed by Gluckman (this symp.) who combined TBR with shielding of both lungs (400 rad + 400 rad lung shielded) and cyclo in an attempt to prevent take failure and pneumonitis in aplastic patients. In all of 16 patients the MHC matched bone marrow graft took, but there were still 3 deaths from interstitial pneumonitis (i.p.) and the incidence of lethal GVHD was high at 32%. The latter observation suggests that at least some of the i.p. is not based on radiation damage to the lung but rather related to immunodeficiency as a consequence of GVHD, which mechanism would also explain the very low incidence of i.p. in leukemic patients conditioned with TBR plus cyclo and grafted with bone marrow from an identical twin donor. Fractionated TBR to a total dose of 3000 rad was reported to permit a take of MHC mismatched marrow in dogs (Kolb, this symp.). An improved conditioning in rats was obtained by Santos et al. (this symp.) by a combination of busulfan and cyclo.

The various presentations involving irradiation demonstrated a regrettable lack of dosimetric standardization, which makes it difficult to compare the various regimens and in fact carries the risk of erroneous dose calculations, with fatal consequences. Doses were presented in R units where clearly rad was needed and there is no way to evaluate the reliability of the actual dose measurements performed in the various centers. In addition, no attention was paid to the significant differences in RBE of the various radiations employed. Much more attention to these important variables is needed in the future (Table 1).

Table 1. Radiation dose standardization

Röntgen to *RAD* 250 kV X ray *Low* dose rate to higher dose rate	
RBE γ	= 0.85
R→rad	= 0.87
3–5 rad/min → 15–20 rad/min	= 0.85
$0.85 \times 0.87 \times 0.85$	= 0.63
Example: 1200 Rγ at 4 R/min = equivalent to 750 rad 250 kV X rays at 20 rad/min	

II. Graft-versus-host Disease

This complication continues to be of grave concern to bone marrow transplant teams because even with the best possible donor matching, i.e. MHC identical siblings, it occurs in about 50% of the successful transplant takes and in half of the patients with GVHD it runs a fatal course. Although quite obvious, it has to be stressed that GVHD can only develop when a complete take of bone marrow is

achieved. Partial takes resulting in incomplete chimerism carry less risk of GVHD, which makes it attractive to achieve a better understanding of the conditions which favour these partial takes.

III. Mechanisms of Induction and Prevention of GVHD

Acute GVHD develops within the first 2 weeks after transplantation and is usually fatal. It is caused by the transfer of immunocompetent T ly of the allogeneic donor, therefore any procedure which reduces the ratio T ly/HSC tends to diminish the risk of acute GVHD. The bone marrow of primates contains a high T ly/HSC ratio and is therefore more dangerous than rodent bone marrow, in which this ratio is so low that acute GVHD does not usually develop following H2 incompatible bone marrow transplantation. In dog bone marrow this ratio is intermediate between that of primates and rodents. Hence, there is no acute GVHD following grafting of MHC identical sibling marrow in the dog. Acute GVHD can be induced by grafting lymph node cells along with the bone marrow in rodents and dogs, or by grafting spleen cells in mice. In the latter species the spleen contains both T ly and HSC. Conversely, primate bone marrow which has been depleted of T ly, e.g. by density centrifugation, no longer induces acute GVHD even when MHC mismatched bone marrow is employed.

When H2 mismatched bone marrow of rodents is successfully transplanted GVHD develops at a later time, that is starting at about 1 month after transplantation and it runs a slower course than acute GVHD, therefore this more protracted form of GVHD is called the delayed type. In thymectomized recipients delayed type GVHD does *not* develop, which suggests that this type of GVHD is due to the presence of immature T cells which require the thymus to develop into cytotoxic T cells. We have previously reported that removal by density centrifugation of the few T ly from H2 incompatible bone marrow or fetal liver cells, does not modify the development nor the course of delayed type GVHD [10].

At this meeting several participants reported an abolishment of delayed type GVHD in H2 incompatible transplants following pretreatment of the bone marrow or of the recipient. Dexter (this symp.) showed that long-term culture of bone marrow, which extinguishes lymphocytes and leaves HSC (CFU-s) intact and in fact multiplying, prevents the delayed type GVHD in a parental donor to F_1 hybrid mouse combination. He has obtained, but did not present, similar results with interstrain H2 incompatible transplants. Müller-Ruchholz et al. (this symp.) reported the abolishment of both acute and delayed type GVHD in rats when bone marrow of H2 incompatible donors was pretreated in vitro with specific antilymphocyte serum (SAL), while pretreatment with specific anti-T cell serum did prevent acute GVHD but not delayed type GVHD. Rat bone marrow which was pretreated with SAL restored lethally irradiated mice which did not subsequently develop GVHD. Finally, Thierfelder et al. (this symp.) could prevent delayed type GVHD in mice after sensitization of the recipients against T cell antigens (but not H2 antigens) of the H2 different donor.

There are several difficulties in interpreting these experiments, in that both Dexter (in mice) and Müller-Ruchholz (in rats) mention the occurrence of acute

GVHD in recipients of H2 incompatible marrow, a phenomenon that we have, so far, failed to observe. But apart from this discrepancy, our group has not been able to abolish delayed type GVHD in mice by removing T lymphocytes from the bone marrow with the technique of density centrifugation. If we consider the maturation scheme of the T ly series which is presently employed by most authors (Fig. 1), the difference between in vitro culture of bone marrow, SAL treatment and anti-donor T antigen sensitization of recipients on the one hand and physical removal of T ly on the other hand, could be that the former 3 treatments inactivate a prethymic precursor T cell and the latter one a post-thymic precursor T cell. However, this hypothesis does not at first sight explain the observation that thymectomized mouse recipients do not develop delayed type GVHD following bone marrow grafting, as the bone marrow contains post-thymic precursor cells. These cells require thymus humoral factor to become responsive to antigen. Since removal of T lymphocytes from bone marrow does not abolish the delayed type GVHD, it seems more likely that density gradient separation leaves the post-thymic precursor cell in the HSC fraction and only removes T lymphocytes. The lower incidence of delayed type GVHD following fetal liver cell transplants as compared to bone marrow transplants suggests that fetal liver contains less post-thymic precursors. It remains unsolved whether SAL treatment and the Dexter culture system merely remove post-thymus precursor cells or in addition prethymic precursors, but it seems likely that immunization of the recipient against T cell antigens merely inactivates the post-thymic precursors, since prethymic precursors probably have not yet acquired the specific antigenic properties of T cells.

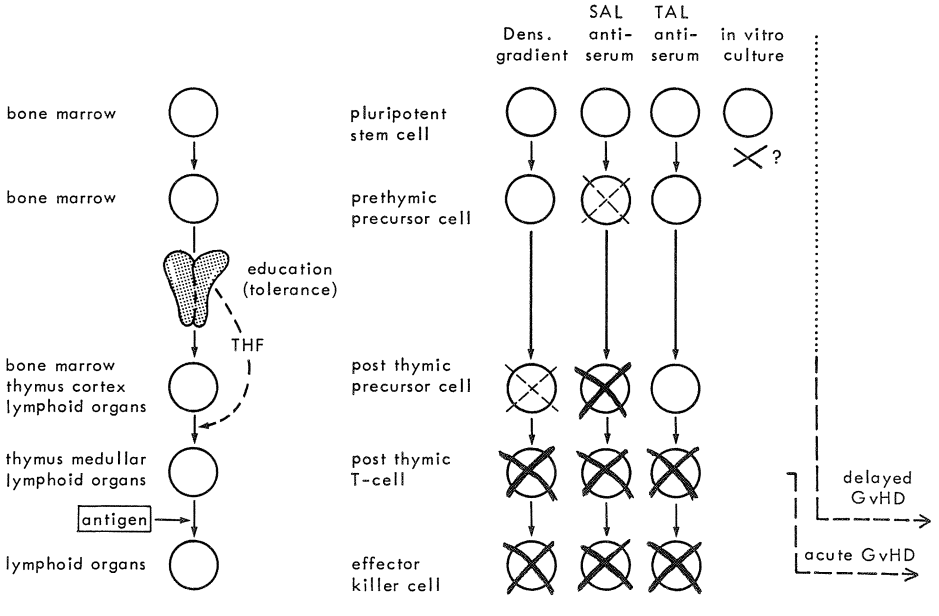


Fig. 1. Maturation scheme of the T lymphocyte series depicting possible mechanisms of GVHD prevention by various in vitro methods. ⊗ Cells most likely eliminated; ⊗ cells possibly also eliminated

These various results then leave us for the present with the proposition that delayed type GVHD is induced by post-thymic precursor cells which require the thymus for differentiation into T lymphocytes and that acute type GVHD is induced by T lymphocytes, which only require stimulation with specific antigens to start reacting against host tissues. It has also been demonstrated that delayed type GVHD does not develop in germfree or Enterobacteriaceae-free mice (3, 8) and we have provided evidence for cross-reacting antigens in the gramnegative microflora of the mouse gut with antigens of mouse intestinal epithelial cells [2]. Acute GVHD is somewhat but not completely diminished by removal of the microflora, which indicates that stronger antigen supply is required to initiate the GVH reaction when T ly are slowly generated out of post-thymic precursor cells than in the case of a sufficiently large supply of T ly being available.

IV. Histoincompatibility factors

The fact that MHC identical donor bone marrow does induce GVHD continues to stimulate research into the role of minor histocompatibility antigens. Halle-Panenko (this symp.) provided evidence for this important issue in mice. Some of these minor histocompatibility antigens are located on the Y-chromosome and appear to be relevant to human [18, 4] and canine [22] MHC identical transplants. Female to male and female to female grafts carry a higher incidence and severity of GVHD than male to male and male to female combinations. The significance of these observations is that in addition to the postulated control of GVHD on the Y-chromosome, female donors – for still ill-understood reasons – lead to more aggressive GVHD. This might be another example of the more aggressive behaviour of female lymphocytes, as is also evident from the higher incidence of autoimmunity in females. Perhaps a sobering thought for male chauvinists; anyway it indicates that if a choice can be made between a male or female donor for clinical bone marrow transplantation, the male donor is to be preferred.

Low MLC reactions do not seem to be a crucial requirement for successful takes and controllable GVHD in human patients, as was shown by the SCID data from the International Bone Marrow Transplant Registry [4], the data from the UCLA bone marrow transplant group [12] and the report of Dupont (this symp.). A similar conclusion may be drawn from published data in dogs by Kolb [9], Storb [19] and Vriesendorp [23]. In rhesus monkeys, Wagemaker et al. have found that A and B identity among unrelated donor-recipient combinations decreases the risk of GVHD (Table 2). It is noteworthy that the purified stem cell preparations employed in these studies took well when matched for A+B, so that clinical exploration of similar transplants seems to be justified, provided that the risk of delayed type GVHD is minimized by bacteriological decontamination.

V. Suppressor cells

While stable chimerism in the absence of GVHD as well as recovery from delayed type GVHD were previously ascribed to the development of specific immunological tolerance on the part of the donor T cells, current immunological concepts

Table 2. Effect of matching for components of MHC on results of allogeneic stem cell transplantation in Rhesus monkeys[a]

Donor-recipient combination	MHC identity MLC	SD	Take	Median survival time recipients with take (days)	Fatal GVH
Unrelated	≠	≠	3/10	27	3/3
Unrelated	=	≠	3/3	<21	3/3
Unrelated	≠	=	6/7	88	2/6[b]
Related 1 MHC haplotype different	≠	≠	5/8	28	4/5[b]
Related MHC identical	=	=	5/5	>120	0/5

[a] Wagemaker and van Bekkum, 1979, unpublished observations; 850 rad X ray TBR, CFU-c equivalent of 4×10^8 bm cells.kg^{-1} from fractions of a discontinuous albumen gradient

[b] Remaining animals died from complications of late immunodeficiencies

favour a role of T suppressor cells in these situations. Tutschka and Santos found indeed that chimeric peripheral blood lymphocytes could decrease the incidence of GVHD in rats being grafted with allogeneic marrow [21]. Already in 1962 van Putten [13] described a similar phenomenon in mice using spleen cells of established chimeras, which was at that time interpreted as follows: "It is assumed that the tolerant cells compete with the injected non-tolerant cells, and that this competition is probably responsible for a decreased proliferation of the non-tolerant lymphoid cells, thereby suppressing their lethal effect." In our own rat strain combination BN → WAG spleen cells of chimeras did suppress the acute GVHD induced by fresh BN spleen cells to a limited extent only and the effective ratio suppressor (chimeric) cells/inducer cells had to be quite high. In the present meeting suppressor cells were discussed in relation to the presentation by Bacigalupo et al. (this symp.), who demonstrated suppression of B cell activity by peripheral T cells of grafted patients. Clearly the precise role of the suppressor T cell subpopulation in bone marrow transplantation remains to be clarified.

C. Clinical Bone Marrow Transplantation

I. SCID Patients

Apart from the already mentioned report on mixed chimerism in 3 patients by Niethammer, two presentations provided more detailed immunological information in grafted patients. Touraine (this symp.) demonstrated cooperation between T cells of the donor and B cells of the recipient in patients treated with fetal liver cells and thymus cells from the same donor and similar results were provided by Griscelli (this symp.).

II. Aplasia

In this condition the problems of graft rejection can be overcome by a variety of different approaches: TBR with lung shielding (Gluckman), TLN irradiation (Kersey) and the conventional cyclo conditioning supplemented with 4×10^8 viable mononuclear peripheral blood cells from the donor (Storb et al., this symp.). The latter procedure has been used in 70 patients in Seattle and reduced the rejection rate from the previous 32% to 13%, resulting in an overall survival of 75%. There was no increased incidence of GVHD following this treatment, contrary to what might be expected on the basis of numerous animal experiments involving the addition of lymphoid cells to the bone marrow graft. In fact, Rieder et al. [15] showed that GVHD was significantly increased in lethally irradiated dogs given leukocyte transfusions in addition to bone marrow from MHC identical siblings. They also showed a much improved take of MHC identical marrow in dogs conditioned with cyclo when donor leukocytes were added to the graft. Surprisingly, in this group GVHD was not increased by the leukocyte transfusions. Storb et al. attribute the take rate improval to the addition of HSC contained in the peripheral leukocytes, but this seems unlikely as the content of HSC in peripheral blood is such that the total number of HSC grafted was increased by only a few percent at most. A more likely postulate is that the leukocytes add to the abolition of circulating anti-donor antibodies in previously sensitized patients and that the T lymphocytes become inactivated in the process, thus preventing their reaction in GVHD. However, this would still leave additional GVHD to be expected in non-sensitized recipients.

Clearly, it is important to elucidate the intriguing effects of blood leukocytes in animal experiments.

Fliedner (this symp.) reported progress in the attempts of his group to utilize peripheral blood leukocytes for the eventual treatment of aplastic patients. In lethally irradiated dogs, $2–4 \times 10^4$ CFU-c/kg body weight of peripheral blood cells are sufficient to restore hemopoiesis when MHC identical donors are employed. The majority of the recipients die with GVHD (which is usually mild and non-lethal in dogs receiving marrow from similar donors), but this could be prevented by grafting only fraction 2 cells of the discontinuous albumen gradient as originally described by Dicke et al. [6].

The advantages of more dependable conditioning regimens as now seem to be emerging are that much more emphasis can be laid on the prevention of GVHD. The procedures presently available: lower total bone marrow cell numbers, removal of T lymphocytes by density gradient separation, the pretreatment of the bone marrow with specific antilymphocyte serum in vitro as well as the posttransplantation treatment of the recipient with methotrexate, all tend to reduce the numbers of available donor stem cells, which tends to diminish the takeability of the bone marrow graft when conditioning has been marginal.

An interesting finding in successfully treated patients with aplastic anemia was reported by de Koning (this symp.). In patients who improved under conventional treatment as well as in those who recovered following an MHC identical bone marrow graft, the proportion of CFU-c in the bone marrow remained subnormal and frequently very low, even when determined several

years after adequate restoration of peripheral blood values had been achieved. It would be interesting to know if such decreased CFU-c levels will be observed in long-term survivors by other transplant teams and in experimental animals.

III. Leukemia

One of the most encouraging developments in the treatment of acute leukemia with TBR, cyclo and bone marrow transplantation was reported by Storb (this symp.) for the Seattle marrow transplant team. Since 1976 they have been treating leukemia patients in remission (ALL second or subsequent, AML first or subsequent) reasoning that one of the most frequent causes of failure – the recurrence of the leukemia, might be reduced when the patient is treated at a time when the tumor cell load is much smaller than when the patient is in relapse. So far, 43 patients have been treated in this way and present survival stands at about 50% which is significantly higher than comparable data for patients transplanted in relapse. It seems even more essential than before that those medical centers which treat large numbers of leukemic patients with either bone marrow transplantation when donors are available or with current chemotherapy when a donor cannot be found, set up prospective therapeutic trials to ascertain the value of transplantation versus the slowly improving results of intensive chemotherapy.

Attempts to improve on the high leukemic relapse rate were also made by Santos et al. (this symp.) by adding busulfan to the standard cyclo regimen, but the results so far are insufficient to allow any evaluation. From the laboratory came an interesting progress report by Bortin et al. (this symp.) who found that allosensitization of mouse bone marrow donors CBA ($H2^k$) against spleen and thymus cells from RF or B10Br mice (both $H2^k$) increased the anti-leukemic effect of their spleen cells in AKR leukemic mice, without increasing the GVHD. Although still far from clinical application, the further elucidation of the effect might be helpful in developing specific anti-leukemic immunization schedules.

IV. Autologous Bone Marrow Transplants

The recent revival of the use of autologous transplants brought about by the pioneer work of Dicke et al. resulted in 5 presentations at this conference. Dicke (this symp.) reported on the treatment of leukemic patients in relapse with TBR, piperazinedione and autologous bone marrow. He obtained 50% CR so far in 21 patients. His attempts to remove leukemic clonogenic cells from the remission bone marrow before reinfusion by density gradient centrifugation were not yet evaluable. Attempts to selectively inactivate residual leukemic cells in autologous remission marrow with anti-leukemic stem cell sera were reported by Kaizer et al. (this symp.) in rodents and by Netzel et al. (this symp.) in human bone marrow, both employing heterologous sera. The results were encouraging in that these sera appeared to be non-toxic for normal stem cells. These approaches are not the only ones which are presently being pursued. In Rijswijk, Martens et al. [11] have obtained promising results by using electrophoretic cell separation on neuramini-

dase treated cells from myeloid leukemic BN rats and it does not seem that these various approaches have yet exhausted all possibilities.

Schaefer et al. (this symp.) and Kaizer et al. (this symp.) addressed themselves to the question of whether CFU-c content and proliferative activity of bone marrow were affected in remission bone marrow of patients treated for leukemia and solid tumors respectively with chemotherapeutic agents. Their results are encouraging in the sense that the impairment of CFU-c and proliferation was not statistically significant.

D. Conclusion

In both the fields of experimental and clinical bone marrow transplantation steady and exciting progress was being reported. The two major barriers: graft rejection and GVHD are successfully being reduced, meaning that the indications for allogeneic bone marrow transplantation are extended gradually. Eradication treatment of leukemia has made a major step forward by the introduction of bone marrow transplantation in remission patients by the Seattle group. The decreased relapse rates so far observed provide a strong support for the classical notion of Skipper et al. [17] that eradication success is inversely dependent on tumor cell load. Autologous bone marrow transplantation is clearly out of the experimental area and its usefulness has to be evaluated in the clinic from now on. The same holds for much work on prevention and abolition of GVHD, but the elucidation of its mechanisms still require a lot of basic research. At the same time the limitations for clinical bone marrow transplantation are moving now to the majority of patients who do not have an MHC identical sibling donor. Selection of suitable non-related donors has shown considerable advance in rhesus monkeys and some of these findings are presently ready for clinical exploration in SCID patients where the lack of sibling donors is most obvious.

References

1. Bekkum, D. W., van: Immunological reactivity and specific tolerance of the graft in radiation chimeras and the influence of the thymus. In: La greffe des cellules hématopoïétiques allogéniques, p. 381. Paris: Editions du C.N.R.S. 1965
2. Bekkum, D. W., van, Knaan, S.: Role of bacterial microflora in development of intestinal lesions from graft-versus-host reaction. J. Natl. Cancer Inst. *58*, 787 (1977)
3. Bekkum, D. W., van, Roodenburg, J., Heidt, P. J., Waaij, D., van der: Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of the intestinal microflora. J. Natl. Cancer Inst. *52*, 401–404 (1974)
4. Bortin, M. M., Rimm, A. A. for the Advisory Committee of the International Bone Marrow Transplant Registry: Severe Combined Immunodeficiency Disease. Characterization of the disease and results of transplantation. JAMA *238*, 591–600 (1977)
5. Dexter, T. M., Moore, M. A. S., Sheridan, A. P. C.: J. Exp. Med. *145*, 1612 (1977)
6. Dicke, K. A., Hooft, J. I. M. van, Bekkum, D. W. van: The selective elimination of immunologically competent cells from bone marrow and lymphatic cell mixtures, II. Mouse spleen cell fractionation on a discontinuous albumin gradient. Transplantation *6*, 562–570 (1968)

7. Dicke, K. A., Platenburg, M. G. C., Bekkum, D. W., van: Colony formation in agar: in vitro assay for hemopoietic stem cells. Cell. Tissue Kinet. *4*, 463–477 (1971)
8. Heit, H., Wilson, R., Fliedner, T. M., et al.: Mortality of secondary disease in antibiotic-treated mouse radiation chimeras. In: Heneghan, J. B. (Ed.): Germfree Research: Biological Effects of Gnotobiotic Environment, pp. 477–485. New York and London: Academic Press 1973
9. Kolb, H. J., Rieder, I., Grosse-Wilde, H., et al.: Marrow grafts in LD-SD typed dogs treated with cyclophosphamide. Transpl. Proc. *8*, 555 (1976)
10. Löwenberg, B., Zeeuw, H. M. C. de, Dicke, K. A., Bekkum, D. W. van: Nature of the delayed graft-versus-host reactivity of fetal liver cell transplants in mice. J. Natl. cancer Inst. *58*, 959 (1977)
11. Martens, A. C. M.: Experimental Hematology (in press)
12. Opelz, G., Gale, R. P., Fey, S. A. et al., and the UCLA Bone Marrow Transplant Team: Significance of HLA and non-HLA antigens in bone marrow transplantation. Transpl. Proc *10*, 43 (1978)
13. Putten, L. M. van: Competition between lymphoid cells from different sources in radiation chimeras. Transplantation Bulletin *29*, 421–423 (1962)
14. Reimann, J., and Burger, H.: British J. Haematol. (in press)
15. Rieder, I., Kolb, H. J., Schaffer, E., Kolb, H., Gross-Wilde, H., Scholz, Thierfelder, S.: Leukocyte transfusions for the modification of host-versus-graft reactions in dogs. In: Baum, S. J., Ledney, G. D. (eds.): Experimental Hematology Today, pp. 101–109. Berlin, Heidelberg, New York: Springer 1978
16. Seller, M. J.: J. Med. Genetics 7, 305 (1970)
17. Skipper, H. E.: Cancer *21*, 600 (1968)
18. Storb, R., Prentice, R. L., Thomas, E. O.: Treatment of aplastic anemia by marrow transplantation from HLA identical siblings. J. Clin. Invest. *59*, 625–632 (1977)
19. Storb, R., Weiden, P. C., et al.: Failure of engraftment and graft-versus-host disense after canine marrow transplantation. Two phenomena linked to but not exclusively determined by known antigens of the major histocompatibility complex. Transpl. Proc. *10*, 113 (1978)
20. Strober, S., Slavin, S. et al.: Transpl. Proc. *11*, 1032 (1979)
21. Tutschka, P., Schwerdtfeger, R., Slavin, R., Santos, G.: Mechanism of donor to host tolerance in rat bone marrow chimeras. In: Baum, S. J., Ledney, G. D. (eds.): Experimental Hematology Today. pp. 191–197. Berlin, Heidelberg, New York: Springer 1977
22. Vriesendorp, H. M., Bijnen, A. B., Kessel, A. C. M., van, Obertop, H., Westbroek, D. L.: Minor Histocompatibility systems in dogs. In: Baum, S. J., Ledney, G. D. (Eds): Experimental Hematology Today, pp. 109–117. Berlin. Heidelberg. New York: Springer 1978
23. Vriesendorp, H. M., Bijnen, A. B., Zurcher, C., Bekkum, D. W., van: Donor selection and bone marrow transplantation in dogs. In: Kissmeyer-Nielsen, F. (Ed.): Histocompatibility Testing, pp. 963–971. Copenhagen: Munksgaard 1975
24. Welling, W., Vos, O., Weyzen, W. W. H., Bekkum, D. W., van: Identification and follow-up of homologous and heterologous bone marrow transplants in radiation chimeras. Int. J. Radiat. Biol. *1*, 143 (1959)
25. Zinkernagel, R. M., Callahan, G. N., et al.: J. Exp. Med. *147*, 882 (1978)

Anaesthesiologie und Intensivmedizin Anaesthesiology and Intensive Care Medicine

95 Mobile Intensive Care Units
Advanced Emergency Care Delivery Systems
Edited by R. Frey, E. Nagel, P. Safar
Assistant Editors: P. Rheindorf, P. Sands
1976. 67 figures. XV, 271 pages
(61 pages in German)
ISBN 3-540-07561-5

98 Intraaortale Ballongegenpulsation
Experimentelle Untersuchungen zur Frage des Wirkungsspektrums und der klinischen Indikation
Von E. R. de Vivie
1976. 42 Abbildungen, 8 Tabellen. X, 96 Seiten
ISBN 3-540-07776-6

100 Anaesthesie und ärztliche Sorgfaltspflicht
Von H. W. Opderbecke
1978. 1 Tabelle. IX, 124 Seiten
ISBN 3-540-08976-4

101 Myokarddurchblutung und Stoffwechselparameter im arteriellen Blut bei Hämodilutionsperfusion
Von D. Regensburger
1976. 20 Abbildungen, 14 Tabellen. VII, 75 Seiten
ISBN 3-540-07877-0

102 Coronarinsuffizienz, Pathophysiologie und Anaesthesieprobleme bei der Coronarchirurgie
Bericht des Workshops am 23. und 30. Juni 1975 in Düsseldorf/Amsterdam
Herausgegeben von M. Zindler, R. Purschke
1977. 79 Abbildungen, 19 Tabellen.
XIII, 166 Seiten
ISBN 3-540-08015-5

103 Fettemulsionen in der parenteralen Ernährung
Symposion im Juni 1976 in Stockholm
Herausgegeben von A. Wretlind, R. Frey, K. Eyrich, H. Makowski
1977. 95 Abbildungen, 33 Tabellen. X, 222 Seiten
ISBN 3-540-08104-6

104 Die akute normovolämische Hämodilution in klinischer Anwendung
Von A. J. Coburg
1977. 21 Abbildungen, 17 Tabellen. XI, 89 Seiten
ISBN 3-540-08025-2

105 Lungenveränderungen während Dauerbeatmung
Von H. Reineke
1977. 26 Abbildungen, 7 Tabellen. VII, 56 Seiten
ISBN 3-540-08101-1

106 Etomidate
An Intravenous Hypnotic Agent
First Report on Clinical and Experimental Experience
Edited by A. Doenicke
1977. 59 figures, 16 tables. XI, 155 pages
ISBN 3-540-08485-1

107 Die kontrollierte Hypotension mit Nitroprussidnatrium in der Neuroanaesthesie
Von K. Huse
1977. 9 Abbildungen, 38 Tabellen. IX, 98 Seiten
ISBN 3-540-08218-2

108 Transcutane Sauerstoffmessung
Methodik und klinische Anwendung
Von K. Stosseck
1977. 31 Abbildungen, 6 Tabellen. VIII, 68 Seiten
ISBN 3-540-08481-9

109 20 Jahre Fluothane
Herausgegeben von E. Kirchner
1978. 151 Abbildungen, 56 Tabellen.
XVIII, 343 Seiten. (18 Seiten in Englisch)
ISBN 3-540-08602-1

110 Neue Untersuchungen mit Gamma-Hydroxibuttersäure
Herausgegeben von R. Frey
1978. 63 Abbildungen, 34 Tabellen. XIII, 149 Seiten
(79 Seiten in Englisch)
ISBN 3-540-08724-9

Springer-Verlag
Berlin
Heidelberg
New York

Anaesthesiologie und Intensivmedizin Anaesthesiology and Intensive Care Medicine

111 Anaphylaktoide Reaktionen
nach Infusion natürlicher und künstlicher Kolloide
Von J. Ring
Geleitwort von K. Messmer und R. Frey
1978. 65 Abbildungen, 84 Tabellen. XV, 202 Seiten
ISBN 3-540-08753-2

112 Kreislaufproblematik und Anaesthesie bei geriatrischen Patienten
Von G. Haldemann
1978. 24 Abbildungen, 3 Tabellen. VIII, 55 Seiten
ISBN 3-540-08785-0

113 Regionalanaesthesie in der Geburtshilfe
Unter besonderer Berücksichtigung von Carticain
Herausgegeben von L. Beck, K. Strasser, M. Zindler
1978. 19 Abbildungen, 24 Tabellen. IX, 94 Seiten
ISBN 3-540-08828-8

114 Zur funktionellen Beeinflussung der Lunge durch Anaesthetica
Von B. Landauer
Geleitwort von E. Kolb
1979. 53 Abbildungen, 61 Tabellen. XV, 155 Seiten
ISBN 3-540-09042-8

115 Zum Problem der Aspiration bei der Narkose
Intraluminales Druckverhalten im Oesophagus-Magen-Bereich
Von G. Sehhati-Chafai
1979. 27 Abbildungen, 55 Tabellen. X, 99 Seiten
ISBN 3-540-09162-9

116 Acute Care
Based on the Proceedings of the Sixth International Symposium on Critical Care Medicine
Edited by B. M. Tavares, R. Frey
1979. 133 figures, 100 tables. XVI, 345 pages
ISBN 3-540-09210-2

117 Der Einfluß von Anaesthetica auf die Kontraktionsdynamik des Herzens
Tierexperimentelle Untersuchungen
Von K.-J. Fischer
1979. 181 Abbildungen, 33 Tabellen. XII, 276 Seiten
ISBN 3-540-09143-2

118 Dobutamin
Eine neue sympathomimetische Substanz
Herausgegeben von H. Just
1978. 56 Abbildungen, 6 Tabellen. XI, 81 Seiten
ISBN 3-540-09077-0

119 Sympathico-adrenerge Stimulation und Lungenveränderungen
Von G. Metz
1979. 40 Abbildungen, 11 Tabellen. VIII, 90 Seiten
ISBN 3-540-09168-8

120 Äthylenoxid-Sterilisation
Von E. G. Star
1979. 2 Abbildungen, 4 Tabellen. VIII, 43 Seiten
ISBN 3-540-09294-3

121 Zur Herzwirkung von Inhalationsanaesthetica
Der isolierte Katzenpapillarmuskel als Myokard-Modell
Von H. P. Siepmann
1979. 14 Abbildungen, 5 Tabellen. VIII, 63 Seiten
ISBN 3-540-09230-7

122 Coronare Herzkrankheit
Physiologische, kardiologische und anaesthesiologische Aspekte
Weiterbildungskurs für Anaesthesieärzte am 10. Juni 1978 in Wuppertal
Herausgegeben von J. Schara
1979. 61 Abbildungen, 15 Tabellen. IX, 97 Seiten
ISBN 3-540-09416-4

123 Pathologische pulmonale Kurzschlußperfusion
Theoretische, klinische und tierexperimentelle Untersuchungen zur Variabilität
Von H. Kämmerer, K. Standfuss, E. Klaschik
1979. 23 Abbildungen, 8 Tabellen. VIII, 71 Seiten
ISBN 3-540-09498-9

Springer-Verlag
Berlin
Heidelberg
New York