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Techniques for Separation and Selection of Antigen Specific Lymphocytes[1]

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I. Introduction

The immune system consists of a complex array of cells in various stages of differentiation and forms of specialization. Many different subpopulations of lymphocytes have been distinguished according to physical, biochemical, physiologic, and functional properties. There are two major classes of lymphocytes; B cells and T cells. The repertoire of receptors for antigen on mature cells of both classes is very diverse, each individual cell and its progeny being committed and restricted to synthesize molecules with identical antigen-combining sites. This restriction in specificity has been predicted in the clonal selection theory and was first demonstrated at the level of antibody-secreting cells and subsequently also at the level of unstimulated lymphocytes. Techniques which

allowed the elimination or isolation of cells expressing specific receptors provided the most convincing experimental evidence for the clonal selection theory.

The following techniques will be described: 1) isolation or elimination of lymphocytes expressing specific receptors (Sect. II), 2) positive and negative selection of antigen-reactive lymphocytes in vivo (Sect. III) or in vitro (Sect. IV), and 3) isolation of lymphocyte clones in vivo (Sect. V.B) or in vitro (Sect. V.C). These techniques were applied in many studies on the nature of antigen receptors of B- and T-lymphocytes and in studies on the requirements for activation, growth, and differentiation of antigen-specific lymphocytes.

In this review we describe the achievements of various techniques as well as their difficulties and limitations which have often been understated. Hopefully, this will help the reader to choose or to develop the technique that best suits his purpose.

II. Specific Lymphocyte Separation via Antigen Binding

A. Introduction

The possibility of detecting antigen binding to single lymphocytes, which were not previously in contact with this antigen, opened up new possibilities for testing the clonal selection theory. *Naor* and *Sulitzeanu* (1967) first demonstrated the binding of ^{125}I-labeled albumin to a small fraction of antigen-specific lymphocytes by radioautography. This technique was taken up and improved by many investigators (for review, see *Ada,* 1970; *Roelants,* 1972; *Warner,* 1974; *Diener* and *Langman,* 1975). In addition, a few other techniques were developed for studies on antigen-binding cells (ABC) such as immunocytoadherence of particulate antigens, fluorescence labeling, or binding of antigens that were enzymes followed by detection with substrate (for review, see *Warner,* 1974). Experiments in which ABC were killed or at least inactivated by exposure to highly radioactively labeled antigens strongly suggested that ABC included antibody-forming cell precursors (AFCP) (Sect. II.G).

Antigen-binding studies and hot antigen "suicide" experiments together with a large body of more indirect evidence (*Mäkelä* and *Cross,* 1970; *Nossal,* 1974) convinced most immunologists that 1) antigen receptors of B-lymphocytes are membrane-associated immunoglobulins (M-Ig) and 2) at least the antigen-combining sites of the M-Ig or secreted Ig of one particular B-lymphocyte and its progeny are identical. Further functional analysis of ABC, however, required the development of specific cell separation techniques which allowed not only elimination but also purification of specific lymphocytes. *Wigzell* (1970) described antigen-coated bead columns in which specific B-lymphocytes were retained. However, this first cellular immunoadsorbent technique did not allow the purification of specific lymphocytes, the main difficulties being nonspecific binding and bad recovery of viable antigen-specific cells. Several new techniques were developed which have overcome—at least partly—the initial difficulties (Sect. II.C). However, these techniques failed—with a few exceptions—to separate specific T-lymphocytes. This class of lymphocytes appears to be specialized

to be activated by certain cell surface antigens or any other antigens only in association with these cell surface antigens. Therefore, immobilized living cells (cell monolayers) were often used in attempts to separate specific T-lymphocytes. "Monolayer separation techniques" are described in a separate Section (II.D) since the use of living cells as immunoadsorbents entails many additional difficulties.

In addition to specific lymphocyte separation by binding to insolubilized antigens or cells, alternative methods were developed that allowed separation of specific lymphocytes after binding particulate antigens (rosette separation techniques (Sect. II.E) or fluorescent antigens or antibodies with the aid of a fluorescence-activated cell sorter (Sect. II.F).

All these techniques for separation of specific lymphocytes can in principle be applied to separation of nonantigen-specific lymphocyte subpopulations which are characterized by a particular cell surface component, for instance M-Ig, θ-antigen, Fc receptors and so on. In the following, these nonspecific separation techniques are not described in detail although they share many difficulties with specific cell separation techniques.

B. General

1. Preparation of Single Cell Suspensions

The starting cell populations should be nonaggregating and highly viable since damaged cells and cell aggregates cause severe problems with all cell separation procedures (*Shortman,* 1974). Damaged cells adhere to surfaces (*Shortman* et al., 1971) or to other cells and may release DNA causing further nonspecific "sticking" of cells (*Edelman* and *Rutishauser*, 1974). Fortunately, several very efficient procedures for removal of damaged cells are now available (*Shortman* et al., 1972; *von Boehmer* and *Shortman*, 1973; *Davidson* and *Parish*, 1975). Large aggregates and fine cell debris should be removed by settling out the cell clumps and centrifuging the suspension at low speed (400 *g*, 7 min) to leave fine debris in the supernatant (*Shortman* et al., 1972).

2. Combinations With Other Cell Separation Techniques

A more extensive preseparation of lymphocyte subpopulations containing the specific receptor-bearing cells to be purified is sometimes essential and often helpful to obtain high enrichment of specific cells. Particularly advantageous is the sequential use of the same cell separation technique for the nonantigen-specific prefractionation and the final specific separation.

Moroz and *Kotoulas* (1973), for instance, separated antigen-specific B cells using two glass bead columns in sequence. Cells adhering nonspecifically were first removed in a glass bead column and the effluent cells were then separated according to antigen specificity in a second glass bead column coated with antigen. The advantage of this technique is that specific cells can be enriched in the second column; the disadvantage is that the most adherent subpopulation of antigen-specific cells is already lost in the first column. This problem is

less severe if specific T cells are separated that are less adherent than B cells. Anti-Ig-coated bead columns (*Binz* and *Wigzell*, 1975c; *Okumura* et al., 1977) or dishes (*Taniguchi* and *Miller*, 1977) were used first for T cell enrichment and then the same solid supports coated with antigen for purification of specific T cells.

Elliott and *Haskill* (1973) prepared a fraction of small lymphocytes sedimenting at unit gravity between 3–6 mm/h which were then subjected to a second velocity sedimentation separation after antigen-specific rosettes were formed. *Haskill* and *Marbrook* (1971) combined density gradient and velocity sedimentation separation of spleen cells before and after specific rosette formation. Obviously, different cell fractionation procedures have to be combined to enrich specific receptor-bearing cells in a particular stage of differentiation or form of specialization.

3. Media

A wide variety of suspension media can be used for specific cell separation. However, tissue culture media containing bicarbonate should be avoided in the absence of an appropriate CO_2 pressure, since otherwise the bicarbonate buffer becomes alkaline and can inactivate cells (*Shortman*, 1974). DNase may be added to the medium to prevent cell aggregation and nonspecific binding caused by the release of DNA from damaged cells (*Edelman* and *Rutishauser*, 1974; *Krawinkel* and *Rajewsky*, 1976). Serum is usually not required. NaN_3 may be used to block cell surface receptor movement and shedding during rosette separation (*Elliott* and *Haskill*, 1973) and during FACS separation (*Julius*, personal communication). The presence of NaN_3 in the separation medium does not prevent subsequent functional studies, since its effect (inhibition of the electron transport chain) can be reversed readily by washing the cells in NaN_3-free medium.

4. Temperature

Antigen-specific cell separations are usually performed at 4° C to prevent capping and shedding of cell surface receptors. Antigen binding to M-Ig of B-lymphocytes occurs readily at a low temperature (*Ada*, 1970), while antigen binding to T-lymphocytes may require metabolic energy provided by the cells only at 20°–37° C (*Hämmerling* and *McDevitt*, 1974; *Kennedy* et al., 1975). Therefore, antigen-specific T-lymphocyte separations were often performed at room temperature or at 37° C (see below).

5. Functional Assays

The major goal of specific cell separation techniques in immunology is to investigate the induction, differentiation, and effector function of antigen-specific lymphocytes. The qualitative and quantitative assessment of specific functions of lymphocytes, however, is difficult. So far, single lymphocytes with a particular specific function cannot be identified at the level of immunocompetent precursor

cells nor at the level of effector cells with the exception of antibody-secreting cells (*Jerne* et al., 1963) and more recently killer cells (*Teh* et al., 1977a). The stimulation of antigen-specific precursor cells cannot be studied in most cases in purified cell populations because other cells and/or their products are required besides the antigen. This in turn makes the evaluation of the efficiency of specific cell separation techniques rather difficult. It is conceivable that often the apparent enrichment or depletion of specific lymphocytes in different fractions is in fact due to alterations of regulatory mechanisms. Therefore, simple systems should be chosen. Enrichment or depletion of specific antibody-forming cell precursors (AFCP) for instance should be tested first with T-independent antigens or mitogens which directly induce differentiation into antibody-forming cells (AFC). After establishment of an efficient separation technique, the B cell responses to T-dependent antigens that require more complex functional assay systems may be studied. The failure to separate a specific cell type with a particular technique is obviously only informative if this technique was previously shown to work efficiently in another situation.

A major problem is to determine how many cells in normal and fractionated cell populations respond to a particular antigen. Limiting dilution analysis is so far the only approach to estimate the frequency of antigen-reactive cells and thus to evaluate the efficiency of a particular technique to deplete or enrich antigen-reactive cells. Specifically purified B-lymphocytes have been analyzed quantitatively in such a way by *Nossal* et al. (1976, 1978).

C. Immunoadsorbents

Specific interactions between molecules present in solid and liquid phases are a characteristic feature of living systems. Such reactions may be mimicked in vitro in order to separate by specific adsorption a substance A with affinity for an immobilized substance B (ligand) from other substances and particularly from substances similar to A that are not able to react with B. This principle was first applied by *Campbell* et al. (1951) for the purification of antihapten antibodies. Biospecific adsorption techniques were further developed and soon provided one of the most important tools for the isolation of antibodies, enzymes, and many other species of molecules. The isolation is carried out in two steps: 1) adsorption by contacting the sample with adsorbents and 2) desorption (elution) of the adsorbed material after removal of nonadsorbed material. Similar procedures may also be used for purification of organelles, membranes, or even whole cells bearing specific receptors or antigens on the surface. To this end, immobilized antigens or antibodies may be used (Fig. 1).

There are two major problems with purification of both molecules or cells via specific adsorption techniques: 1) nonspecific interactions of sample material or cells with the adsorbent and 2) the difficulty of eluting specific material or cells bound via multivalent specific and nonspecific interactions. These problems are obviously much more severe for separation of living cells because of their complex surfaces and because of the restriction to conditions that support cell viability.

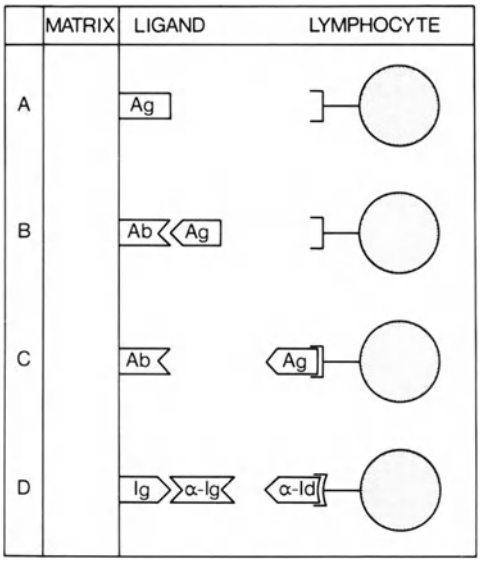

Fig. 1. Immunoadsorbents used for specific cell separation

Most cells including lymphocytes tend to adhere nonspecifically to various surfaces. *Shortman* et al. (1971) distinguished the temperature-independent binding of certain lymphocytes (physical adherence) from the temperature-dependent active adherence of phagocytic cells such as macrophages and polymorphs. The physical or passive adherence of lymphocytes occurs at 4° C as well as at 37° C and is strongly selective for certain subpopulations of lymphocytes. These differential adherence properties of lymphocytes may be useful for separation of nonantigen-specific subpopulations with distinct biologic functions (Table 1). For specific cell separation procedures, however, the nonspecific adherence phenomenon is bothersome for several reasons:

1. Some cells bind to the adsorbents entirely nonspecificly and "contaminate" the specific binding cells if nonspecific elution procedures are used.
2. The binding of a specific cell depends heavily on its nonspecific adherence properties, particularly if its final "trapping" in a column is nonspecific after its flow rate has been reduced selectively by column antigens (*Wigzell*, 1970, 1976).
3. Once a specific cell is bound to the adsorbent, more nonspecific binding sites will be established leading to an essentially irreversible binding.

1. Adsorbent Matrix

The ideal matrix of an immunoadsorbent (solid support) should meet the following demands (*Porath*, 1974, modified for cellular adsorbents):

1. High rigidity and suitable form
2. Insolubility
3. Hydrophilic character
4. Low nonspecific binding capacity for cells
5. Chemical reactivity allowing ligands or spacers to be introduced

Table 1. Nonspecific adherence properties of lymphocyte subpopulations

Adsorbents	More adherent	Less adherent	References
Glass beads	AFC (SRC)		*Plotz* and *Talal* (1967); *Salerno* and *Pontieri* (1969)
	ABC, AFCP, AFC (POL), CTL, lymphoblasts	AFCP (SRC)	*Shortman* et al. (1971, 1972)
	M-Ig pos. cells, ABC, AFC (DNP)	DTH T cells (DNP-GPA), PHA resp. cells	*Rosenthal* et al. (1972)
	AFC (SRC > BSA > ferritin)		*Eckert* and *Pasternak* (1973); *Eckert* et al. (1973a, b)
	PWM resp. cells	T helper cells (SRC), PHA, MLC T cells	*Adams* (1973a, b)
	AFCP (FGG)	AFCP (DNP, SRC)	*Schrader* (1974)
	IgM-AFCP (NIP)	IgG-AFCP (NIP)	*Schlegel* and *Shortman* (1975)
Plastic dishes		AFCP (SRC)	*Mosier* and *Coppleson* (1968);
	AFCP (SRC)		*Loughman* et al. (1974)
Plastic dishes + glass beads		AFCP (SRC), T helper cells (SRC)	*Dutton* et al. (1970); *Hartmann* et al. (1970); *Hirst* and *Dutton* (1970)
Sephadex G-10 beads	AFCP, AFC (SRC)	T helper cells (SRC)	*Ly* and *Mishell* (1974)
	ADC (red cells)	ADC (Sarcoma cells)	*Pollack* et al. (1976)
Nylon wool	CRL	Non-CRL, thymus cells	*Bianco* et al. (1970)
	M-Ig pos. cells	Human T cells	*Eisen* et al. (1972)
	M-Ig pos. cells	T helper cells (KLH), CTLP	*Julius* et al. (1973)
	AFCP (DNP, KLH)	T helper cells (SRC)	*Trizio* and *Cudkowicz* (1974)
	AFCP (TNP, SRC)	E-RFC	*Greaves* and *Brown* (1974)
	Human M-Ig pos. cells	DTH T cells (SRC)	*Cone* et al. (1977)
	T-RFC (SRC)		
Glass wool	ABC, AFC (haptens, SRC), AFC (SRC)		*Loewy* et al. (1975a, b); *Loewy* and *Bussard* (1974)

Table 2. Specific lymphocyte separation using immunoadsorbents

Solid support	Ligands	Separation of	References
Polyurethane foam	Antibody + Antigen	AFC	*Evans* et al. (1969); *Mage* et al. (1969)
Glass or poly metacryl beads (Degalan)	Antigen Hapten	ABC, AFCP	*Wigzell* (1970a, 1976a); *Wigzell* et al. (1971a); *Wigzell* and *Mäkelä* (1970); *Wigzell* and *Andersson* (1971); *Shinghal* and *Wigzell* (1971a); *Iversen* (1973); *Moroz* and *Kotoulas* (1973)
	Antibody	Alloreact. T cells	*Binz* and *Wigzell* (1975c)
Polyacrylamide beads (Bio-Gel)	Hapten	AFC	*Truffa-Bachi* and *Wofsy* (1970) *Wofsy* et al. (1971); *Bellone* et al. (1974)
		AFCP	*Henry* et al. (1972); *Wofsy* (1973)
		Suppr. T cells	*Rubin* (1976)
Agarose beads (Sepharose 2B)	Hapten Antigen	AFCP	*Kishimoto* and *Ishizaka* (1972)
		ABC, AFCP	*Davie* and *Paul* (1970); *Davie* et al. (1971)
(Sepharose 6B)		AFCP, DTH T cells	*Rubin* and *Wigzell* (1973, 1974)
Dextran beads (Sephadex G-25) (Sephadex G-200)	Antibody Antigen	AFCP, CTL	*Scott* (1976a, b)
		Suppr. T cells	*Okumura* et al. (1977)
Hydroxyalkyl methacrylate beads (Spheron)	Antigen	Alloreact. T cells	*Haskova* et al. (1974)
		AFC	*Tlaskalova* et al. (1975)
Gelatin granules	Hapten	ABC	*Gold* et al. (1974)
Nylon fibers	Hapten Antigen	ABC, AFCP	*Edelman* and *Rutishauser* (1974a); *Edelman* (1973a); *Rutishauser* (1975a)
	Antibody	T helper cells	*Hirsch* and *Plescia* (1972)
Meshes	Hapten	ABC	*Kiefer* (1973, 1975)
		T cells (receptors)	*Krawinkel* et al. (1976a)
Polystyrene tubes	Antibody + Antigen	ABC	*Choi* et al. (1974)
Polystyrene dishes	Antigen	Suppr. T cells	*Taniguchi* and *Miller* (1977)

Table 2 (continued)

Collagen-coated dishes	Hapten	DTH T cells	*Webb* et al. (1975)
	Antigen	T helper and suppr. cell	*Maoz* et al. (1976)
		CTL	*Maoz* and *Shellam* (1976)
Gelatin-coated dishes	Hapten	ABC, AFCP	*Haas* and *Layton* (1975); *Haas* (1975); *Nossal* et al. (1976a); *Nossal* and *Pike* (1978)
Gelatin-coated bottles	Antigen	CTL	*Bröcker* and *Sorg* (1977)

Advantages and disadvantages of different matrices are described in detail elsewhere (*Porath*, 1974). Here only some properties of adsorbents are discussed with relevance to specific cell separation techniques. Various types of solid supports are listed in Table 2.

a) Suitable Physical Form

Bead columns are used frequently for cell separation. The beads should have high rigidity not to be easily damagable and an appropriate size. For separation of lymphocytes, rather large beads (>600 µm) were required for good cell recoveries (*Wofsy*, 1973; *Rubin* and *Wigzell*, 1973), although the size of lymphocytes would allow them to pass through much smaller beads. However, columns consisting of 200-µm plastic or glass beads retain a large proportion of cells nonspecifically (*Wigzell* and *Andersson*, 1969), not only by a size filtration effect but also because of insufficient flow rates resulting in an increase of nonspecific binding (*Shortman* et al., 1971).

The total surface of bead columns is usually rather large and the space for binding of cells is not limiting. However, much smaller surfaces are sufficient and probably even more suitable for the purification of a small number of antigen-specific cells. Relatively small surface adsorbents are strung nylon fibers or flat surfaces of dishes or tubes. Here the continuous movement of the cells relative to the adsorbent is of decisive importance not only to allow repeated collisions of all cells with the adsorbents but also to avoid excessive nonspecific binding (see above). Small surfaces have several advantages for specific cell adsorption:

1. The bound cells can be observed in situ with a microscope, and the complete recovery of unbound and bound cells can be controlled by direct inspection.

2. The bound cells can be recovered in relatively small volumes of medium, which facilitates quantitative work with very few cells.

3. The probability of physical trapping of cells as it occurs in edges and corners of a foam or bead column is reduced.

b) Chemical Composition

Glass or plastic beads were used by *Wigzell* and his colleagues for affinity fractionation of lymphocytes (*Wigzell* 1970, 1971, 1976; *Wigzell* and *Andersson*, 1971; *Wigzell* et al., 1971). Up to 50% of lymph node and spleen cells were retained in such columns irrespective of whether antigen was present or not. A slight reduction of nonspecific binding was achieved by pretreating the beads with homologous or heterologous serum (*Shortman*, 1966; *Wigzell* and *Andersson*, 1969; *Wigzell*, 1976).

Major progress was made by *Wofsy* and collaborators, who used affinity columns consisting of large polyacrylamide beads (Bio-Gel). These beads were shown to have low nonspecific adsorption capacity for proteins (*Sanderson* and *Wilson* 1971) and cells (*Truffa-Bachi* and *Wofsy*, 1970). Other materials also appear to be less sticky than glass or plastic, like cross-linked dextran (Sephadex), cross-linked agarose (Sepharose), hydroxy alkyl methacrylate gel beads (Spheron), and gelatin granules or various gelatin-coated surfaces (Table 2). It is conceivable that nonspecific binding of all types of cells to these gels is less pronounced than to glass, plastic, or polyurethan. Tumor cells, for instance, bind much stronger to surfaces of metals, metallic oxides, glass, or plastic materials than to surfaces of organic composition like agarose (*Grinnell* et al., 1972). With lymphocytes, no systematic comparison of nonspecific adherence to various materials has been reported yet. From the available data, it is impossible to determine the material with the lowest nonspecific binding capacity, since nonspecific binding of cells depends not only on the chemical properties of the adsorbent matrix but also on many other parameters, particularly on the contact time between cells and adsorbents. Substantial nonspecific binding of lymphocytes occurs, for instance, to large Sepharose beads (*Rubin* and *Wigzell*, 1973), Sephadex beads (*Ly* and *Mishell*, 1974), or nylon wool (*Julius* et al., 1973) after prolonged incubation at higher temperatures. Differences in nonspecific binding of cells to all the gels mentioned may be negligible under identical separation conditions. However, clear differences may exist after coupling of the ligand (*Wofsy*, 1973).

c) Specific Components of Immunoadsorbents

Antigens, haptens, or specific antibodies can be used as ligands for separation of antigen-specific lymphocytes as schematically shown in Figure 1A–D. Most investigators separating antigen-specific cells used adsorbents coated or covalently coupled with antigens or haptens (Fig. 1A; Table 2). In some cases the antigen was attached to the adsorbents via specific antibodies (*Evans* et al., 1969; *Wigzell* et al., 1972; Fig. 1B). The disadvantage of this technique is that Fc receptor-bearing lymphocytes may bind to the immobilized ag-ab complexes (*Basten* et al., 1972). Experiments by *Wigzell* et al. (1972) suggested that this is not the case. Bead columns coated with anti-Ig-Ig or anti-BSA-BSA did not retain Fc receptor-bearing cells.

The immobilization of antibodies via binding to protein A (*Nash*, 1976), staphylococcus aureus, or protein A-coated red cell monolayers (*Ghetie* and

Sjöquist, 1975) has the advantage that binding of cells to the Fc part does not occur and that the antigen-combining sites are most efficiently located at the adsorbent surface. However, such adsorbents are probably not suitable for specific cell separation because of the large number of antigenic determinants presented by the adsorbents and a high degree of nonspecific binding of lymphocytes (*Ghetie* and *Sjöquist*, 1975). Immobilized antibodies can be used for separation of specific receptor-bearing cells not only after treating the adsorbent but also by treating the cells before the separation with antigen (*Hirsch* and *Plescia*, 1972; *Scott,* 1976a).

The increasing availability of anti-idiotypic antibodies offers a new possibility for specific cell separation. *Binz* and *Wigzell* (1975c) used Ig-anti-Ig-coated columns to separate anti-idiotype-treated lymphocytes, a procedure which is restricted to separation of specific T cells lacking M-Ig (Fig. 1 D). It should be noted here that idiotype-anti-idiotype systems are not necessarily more restricted with regard to affinity ranges and cross-reactivities than other ag-ab systems. Therefore, it should not be expected that more homogenous populations of cells can be separated with the help of anti-idiotypic antibodies.

d) Matrix-Ligand Linkage

Noncovalent physical attachment of most antigens (usually proteins) to glass and plastic (*Wigzell*, 1976; *Taniguchi* and *Miller*, 1977) or nylon (*Edelman* and *Rutishauser*, 1974) appears to be rather stable in physiologic media. However, small amounts of antigen may be released during the cell separation and/or specific receptor-bearing cells may "pick up" antigen from the adsorbent. *Moroz* and *Kotoulas* (1973) demonstrated that radioactively labeled proteins were eluted in readily detectable amounts from polyacrylic (Degalan) bead columns together with passing cells. An additional disadvantage of noncovalent binding procedures, at least for separation of adherent cell types such as B cells, is that matrices to which antigens can be adsorbed in sufficient quantities also tend to bind cells nonspecifically. In general, therefore, covalent binding of ligands to matrices with low nonspecific adsorption capacity should be preferred. Various covalent coupling procedures have been developed in recent years which are not described here in detail (for review, see *Kiefer*, 1978). A few general points are worthwhile to consider:

1. Some chemical reactions tend to destroy the original structure of the adsorbents. Sepharose beads, for instance, are partially split after activation with cyanogen bromide (*Wofsy*, 1971).

2. With some coupling procedures, only a part of the bound ligands is linked through covalent bonds. A substantial amount of protein is noncovalently adsorbed to nylon fibers after coupling with carbodiimide reagents (*Haas*, 1974, unpublished observation; *Edelman* and *Rutishauser*, 1974).

3. From purification of antibodies (*Porath*, 1974) or enzymes (*Steers* et al., 1971) by specific adsorption and elution techniques, it is well-known that small changes in the presentation or spacing of a ligand can have large effects on binding and separation properties of the adsorbent. So far there are no systematic tests on the optimal presentation of ligands for specific cell separation. A simple

test for the specificity and the presentation of the ligand would be to use the adsorbent first for purification of specific antibodies (*Tlaskalova* et al., 1975).

4. Unfortunately, many coupling procedures increase the nonspecific binding capacity of the adsorbent. Loading the matrix with hydrophobic structures or positively charged groups should be avoided when spacers and ligands are coupled (*Truffa-Bachi* and Wofsy, 1970).

e) Epitope Density of the Ligand

The density distribution of "adsorbent centers," specific as well as nonspecific, is progressively important as the size of specific molecules to be separated increases (*Porath,* 1974). Obviously, the epitope density of the ligand is of decisive importance for separations of specific receptor-bearing cells.

Specific binding of a lymphocyte to an adsorbent probably requires simultaneous interactions of several cell surface receptors with several ligands increases tors as well as additional nonspecific interactions. The probability of simultaneous interactions of several cell surface receptors with several ligangds increases with increasing ligand density. Therefore, adsorbents with high epitope density bind more lyphocytes than adsorbents with low epitope density as shown with haptens (*Haas,* 1975). Similarly, more lymphocytes with lower affinity receptors form rosettes if the hapten density on the red cell surface is increased (*Möller,* 1974). Adsorbents with high ligand density may bind some cells bearing receptors with very low affinity for the antigen. Antigen binding to antibodies secreted by such cells or their progeny may not be detectable. The necessity to define the specificity of a cell in functional terms has been discussed in detail before by others, who were confronted with the fact that lymphoid cell populations contained many more cells that bound detectable amounts of an antigen than cells that would respond to this antigen (*Ada*, 1970; *Möller*, 1974).

2. Cell Separation Procedures

a) Contact Time Between Cells and Adsorbents

Prolonged contact between cells and any adsorbent often allows nonspecific binding forces to become very strong (*Grinnell* et al., 1973). Thus, if lymphocytes are allowed to settle onto a surface, a large proportion of them will adhere nonspecifically (Table 1). On the other hand, if the cells move too fast, even specific cells do not bind if the shearing forces are superior to the initial binding forces. For each specific cell separation procedure, conditions should be elaborated that prevent nonspecific binding but still allow binding of specific cells. Sparations in bead columns can be controlled by variation of the flow rates and the bead size.

Adsorbents with relatively small surfaces (like fibers or dishes) have to be moved continuously against the cells. *Edelman* and *Rutishauser* (1974) employed a reciprocal shaker having a 3–4-cm horizontal stroke at 70–80 oscillations per minute or a horizontal rotary shaker at 200 rpm. Such unidirectional agitations have the disadvantage that the cells accumulate around a central line

or on the edge of the dish. This can be avoided by agitating the dishes in two dimensions, on a shaker (moving horizontally through 1.5 cm at 80 cpm) mounted on a rocker moving vertically through 20° at 20 cpm (*Haas* and *Layton*, 1975).

b) Recovery of Unbound Cells

Removal of nonbinding cells from bead columns is simply accomplished by rinsing the columns with medium until no further cells come through. All nonbinding cells should be collected, if their functional capacities are to be tested, since cells that appear later may be a selected population of larger and/or more sticky cells. Some difficulties may arise with the large volumes of medium required for removal of a small number of cells from columns (*Truffa-Bachi* and *Wofsy*, 1970). However, there are similar problems if relatively small surfaces are used as adsorbents. To remove unbound cells from strung nylon fibers, the dishes that accommodate the rings with the fibers have to be immersed in a series of larger vessels containing medium (*Edelman* and *Rutishauser*, 1974). Cells that are trapped between the fiber-supporting plastic rings and the dish or adhere to the dish surface have to be removed by transferring the ring to a new dish. During these procedures, care should be taken not to remove the fibers from the medium because interfacial forces result in immediate release of bound cells. With this procedure it is difficult to maintain sterile conditions and impossible to collect all nonbinding cells. Nylon meshes, however, can be removed from the dishes and washed with medium, a sufficient amount of which always remains in the interstices (*Edelman* and *Rutishauser*, 1974).

Nonbinding cells are readily removed from gelatin-coated dishes by washing several times with fresh medium. A thin layer of medium is always left at the gel surface protecting the bound cells (*Haas* and *Layton*, 1975). The complete removal of unbound cells can be controlled by direct inspection using an inverted microscope. Bound cells are readily distinguishable from unbound cells when the dish is gently agitated.

c) Recovery of Bound Cells (Fig. 2)

Detachment by Mechanical Means (Fig. 2A)

It is obvious that highly enriched populations of specific lymphocytes cannot be recovered by mechanical means from adsorbents that display a high degree of nonspecific binding such as glass or plastic beads (*Wigzell*, 1970), a polyurethan foam (*Mage* et al., 1969), or nylon meshes (*Edelman* and *Rutishauser*, 1974). Release of cells from antigen-coupled taut nylon fibers by plucking with a needle should result in a highly purified cell population since such fibers do not bind a large number of cells nonspecifically under appropriate separation conditions (*Edelman* et al., 1971). However, mechanical release by plucking may produce a lesion in the cell membrane. The viability of "plucked" cells may be as low as 30% but appears to increase sometimes up to 90% after

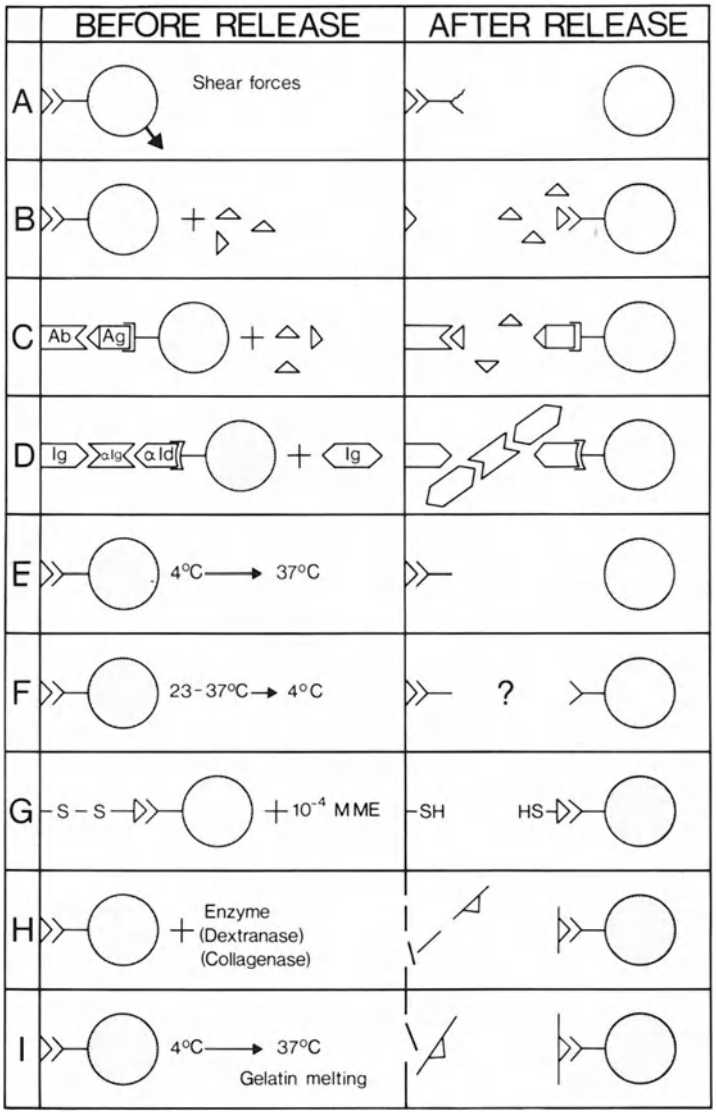

Fig. 2. Methods for recovery of specific cells from immunoadsorbents

incubation for 1 h at 37° C in medium containing fetal calf serum (*Edelman* et al., 1971). We have not observed repair of cell lesions after plucking from nylon fibers (*Haas*, 1975, unpublished results). Probably, the extent of the lesions depends heavily on the number and nature of disrupted binding sites (*Edelman* and *Rutishauser*, 1974; *Mage* et al., 1977). At least in some cases, even nonspecific adherence forces appear to be "stronger than the cell membranes" (*Weiss* and *Coombs*, 1963).

The membrane rupture of single frog lymphocytes after mechanical removal from hapten-coupled nylon fibers was advantageous in experiments in which nuclei of hapten-specific lymphocytes were transferred into enucleated eggs (*Wabl* et al., 1975; *Du Pasquier* and *Wabl*, 1977). Other more gentle detachment procedures should be preferred if the specific cells are separated for functional studies.

Release in the Presence of Competitive Inhibitors (Fig. 2B–D)

The highest degree of purification of specific lymphocytes should be achieved with separation procedures where both the binding and the elution steps are specific. Specific cells should be removed selectively in the absence of strong nonspecific interactions and the presence of soluble "displacer" molecules, in sufficient concentrations, to compete with the ligand. However, the attachment of specific cells to absorbents, even in the most ideal case, usually involves several specific and nonspecific binding sites. This multivalent interaction may explain failures to elute specific lymphocytes in the presence of soluble antigen or hapten from beads (*Wigzell*, 1970; *Rubin*, 1976; *Tlaskalova* et al., 1975), fibers (*Edelman* et al., 1971), or dishes (*Haas*, 1976). However, in some cases, specific cells could be eluted from adsorbents in the presence of specific displacers with or without additional weakening of nonspecific interactions by removal of divalent cations from the eluent medium. In the following, a few reports of successful elution are discussed briefly.

1. *Wofsy* and his collaborators eluted lactoside (lac)-specific lymphocytes from lac-coupled polyacrylamide (Bio-Gel) beads by an excess of free lac in the effluent medium (for review, see *Wofsy*, 1973). The mechanism of the detachment of cells from the beads, however, is not clear. The beads with the bound cells were kept at room temperature for 15 min in order to facilitate equilibrium with the hapten. Therefore, it is conceivable that the detachment of specific cells was supported by capping and shedding of specific receptors (*Kiefer*, 1973; *Krawinkel* und *Rajewsky*, 1976) with the hapten just serving to block further interactions between free receptors and immobilized haptens. *Wofsy* (1973) pointed out that this approach may not be practical in systems characterized by high affinity interactions due to multivalent specific and nonspecific binding forces.

2. *Iversen* (1973) reported that specific cells could be eluted even from sticky plastic beads coated with antigen after prolonged incubation with EDTA and soluble antigen. It is not known whether the specific cells retained their normal function after this treatment.

3. *Choi* et al. (1974) eluted KLH receptor-bearing lymphocytes from (antihapten antibody)-hapten-KLH-coated tubes by an excess of free hapten and the additional action of gravitational forces (Fig. 2C). Surprisingly, the eluted cells carried only relatively small amounts of hapten-KLH. The functional properties of these cells were not studied.

4. *Scott* (1976a) exposed lymphocytes to various fluorescein-conjugated antigens (FLAG) either in vivo or in vitro. The labeled cells were then passed through columns consisting of anti-Fl antibody-coupled Sephadex or Sepharose

beads. Most of the bound cells were eluted when the beads were gently suspended with a Pasteur pipette and free Fl-protein was applied.

5. *De Luca* et al. (1973) tried to separate enzyme-binding cells using substrate-coupled columns. The cells that had bound the enzyme (β-galactosidase) were retained in galactoside columns. However, when free β-galactosidase was used as a modulator (by competing for bead-bound ligand), cells that had bound β-galactosidase were merely retarded resulting in enrichment of enzyme-binding cells in the late eluent fractions. The authors suggested further applications of this technique to the separation of hapten-specific lymphocytes. So far, however, the technique has not been further developed. Some reports on recovery of nonantigen-specific cells from adsorbents should also be mentioned here.

1. Intestinal cells (*Podolsky* and *Weiser*, 1973) or tumor cells (*Killion* and *Kollmorgen*, 1976) were released from *Concanavallin A* (Con A)-coupled nylon fibers in the presence of free sugars.

2. Red cells and lymphocytes were detached from Con A-coupled nylon fibers or Sepharose beads if in addition to the specific displacer (L-methylmannoside) shearing forces were applied by shaking the beads, plucking the fibers, or in the case of red cells, by changes of the cell volume under hypotonic conditions (*Edelman* et al., 1971).

3. *Schlossman* and colleagues described the complete recovery of human surface Ig-bearing lymphocytes from antihuman Ig Fab-conjugated Sephadex beads in the presence of high concentrations of human Ig (10 mg/ml) and EDTA (*Chess* et al., 1974, 1975; *Rocklin* et al., 1974; *Sondel* et al., 1975).

Spontaneous Release After Temperature Shift (Fig. 2E–F)

Kiefer (1973) found that lymphocytes that were bound via M-Ig to hapten-coupled nylon fibers were released within a 2-h incubation period at temperatures above 22° C (Fig. 2E). The release followed M-Ig capping to the site of attachment and was inhibited in the presence of NaN_3. The technique should be well suited to study the conditions leading to specific activation or inactivation of lymphocytes after interaction with antigen-coupled fibers.

The Kiefer technique was applied to the isolation of antigen-specific B and T cell receptors which were left behind at the nylon fibers after the release of the cells (*Krawinkel* and *Rajewsky*, 1976; *Krawinkel* et al., 1977a, b). More recently, another approach has been described by two laboratories. Antigen-specific suppressor T cells were bound at 37° C to antigen-coupled Sephadex beads (*Okumura* et al., 1977) or at room temperature to antigen-coated plastic dishes and released after brief exposure to low temperatures (4° C). The mechanism of this release is not clear yet (Fig. 2F). It is interesting in this context that cytotoxic T-lymphocytes were not released from target cells at 0° C (*Martz*, 1975).

Receptor Destruction

An obvious approach to remove cells from adsorbents is the enzymatic destruction of molecules involved in the binding. Treatment with trypsin is often used

to detach adherent cells such as tumor cells, macrophages, or fibroblasts. The main cell surface components involved in nonspecific interactions with various surfaces appear to be trypsin sensitive as are B-lymphocyte receptors and most likely T-lymphocyte receptors for antigen. Therefore, one would expect that proteolytic enzymes should affect the detachment of specifically adhering lymphocytes, particularly if the ligand would also contain protease-degradable structures. In this case, the receptors, the ligands as well as nonspecific binding sites, would be destroyed by the enzyme. No data are available for such an approach except a brief mention by *Edelman* (1971) that it does not work. For many purposes, other techniques should be preferred which leave the specific cell surface receptors intact.

Ligand Destruction

Enzyme-degradable spacers may be inserted between the matrix and the ligand. *Thomas* and *Phillips* (1973) linked anti-Ig via gelatin onto Sephadex beads. Adherent B cells were recovered by digesting the spacer with collagenase. This principle has not yet been applied for specific cell separation. *Kiefer* (1975) coupled TNP to nylon fiber meshes via a disulfide bridge containing spacer. Fiber-binding cells were then released in the presence of low concentrations of mercaptoethanol ($10^{-4}M$) at 4° C (Fig. 2G). All cell elution techniques based on spacer destruction depend on the absence of strong nonspecific cell-adsorbent interactions.

Matrix Destruction (Fig. 2H)

The most rigorous methods that will mobilize all bound cells, irrespective of the nature of their interactions with the matrix, are based on the complete solubilization of the whole matrix. *Schlossman* and *Hudson* (1973) digested anti-Ig-coupled Sephadex beads with dextranase. Bound B-lymphocytes were liberated but small fibrous residues remained bound to their surface. *Maoz* and collaborators (*Maoz* and *Fuchs*, 1974; *Maoz* et al., 1976; *Maoz* and *Shellam*, 1976) used collagenase-digestable adsorbents such as collagen strings and fibers or dishes coated with collagen or gelatin. The advantage of such adsorbents as compared to Sephadex bead columns is the much smaller amount of matrix material that has to be solubilized. However, even in this case, treatment with collagenase for 90 min at 37° C was required to liberate the majority of the bound cells.

Melting of the Matrix (Fig. 2I)

Gelatin forms an insoluble network at sufficiently high concentrations below a critical temperature. The melting point of a gelatin gel is relatively sharp as is characteristic for network gels that are stabilized by secondary forces such as hydrogen bonds. The gelation and melting characteristics of gelatin are not significantly affected when small haptens are coupled to its lysine residues that are not involved in the gelation process (*Grabar* and *Morel*, 1950). Hapten-

conjugated gelatin has been used as an adsorbent that can be solubilized simply by melting the gel structure at physiologic temperatures (*Rutishauser* et al., 1973; *Maoz* and *Fuchs*, 1974; *Haas* et al., 1974). In all cases, the cells recovered by melting are highly viable and the hapten-gelatin that remained bound to their surface after washing could be removed in some cases by treatment with collagenase (*Haas* and *Layton*, 1975; *Webb* et al., 1975).

3. Applications

a) General Comments on Depletion of Specific Cells

It appears to be a general rule that immunoadsorbent techniques that allow high purification of specific cells are not very efficient in depleting a cell population of specific cells and vice versa. So far, complete depletion of specific AFCP was achieved only with antigen-coupled plastic bead columns of large size (*Wigzell*, 1970). Such columns retain up to 50% of lymphoid cells nonspecifically and careful specificity controls are required to demonstrate that the column antigen was – at least in part – responsible for the complete retention of specific cells. Moreover, in some cases, it is conceivable that the specific depletion is in fact at least partially due to blockade of specific cells by eluted column antigen. This objection is eliminated in cases in which the presence of excess of free antigen during the fractionation was shown to reduce or to abolish the depletion.

Most of the authors who attempted to purify specific lymphocytes did not study the efficiency of their techniques by testing how many specific cells remained in the nonbinding cell population. As discussed earlier, a fine balance between the rate of specific and nonspecific binding has to be established for each separation procedure. For high enrichment of specific cells, conditions are preferred that prevent nonspecific binding as much as possible, usually at the expense of some specific cells that fail to bind.

When spleen cells were fractionated on hapten-gelatin-coated dishes, a substantial proportion of specific cells remained in the nonbinding population. Particularly AFCP activity appeared to be poorly depleted. This may be at least in part due to regulatory mechanisms favoring the in vitro response of smaller numbers of AFCP. However, a significant proportion of ABC also remained in the nonbinding cells, indicating a low efficiency of the gelatin layer separation technique. Several possible explanations for this low efficiency were ruled out (*Haas*, 1976).

1. Space for binding was not limiting.

2. The specific cells that failed to bind were not preferentially low affinity cells.

3. The failure of some specific cells to bind was not due to inhibition by solubilized hapten-gelatin or hapten-gelatin picked up from the gel layer, although careful preparation of the gel layer is required to avoid this effect. It is also conceivable that some specific cells fail to bind because they are trapped in small aggregates formed in the course of the separation. In addition, it cannot be excluded that a certain proportion of AFCP did not express suffi-

cient numbers of surface receptors at the time of the fractionation. Thus, it remains to be established whether highly purified hapten-specific B cells are representative for all hapten-specific B cells or whether they are distinguishable from the specific cells that failed to bind to the adsorbent. Experiments reported by *Fidler* and *Pike* (1977) indicate that the hapten-specific lymphocytes that bind to hapten-gelatin and those that fail to bind are not distinguishable by size differences since all fractions of spleen cells obtained by velocity sedimentation contained an expected proportion of hapten-gelatin binding AFCP.

b) Separation of Specific B Cells

Antigen-Binding Cells (ABC)

Nylon fibers or meshes covalently coupled with various proteins, hapten-protein conjugates, or red cell stromata were used by *Edelman* and collaborators to study ABC (*Edelman* et al., 1971; *Rutishauser* et al., 1972, 1973; *Rutishauser* and *Edelman*, 1972). Numbers of ABC were expressed as total cells bound to the edge of a 2.5-cm fiber segment. Tosyl-BSA-coupled fibers, for instance, bound 130 cells per 2.5 cm from normal *rabbits* and 297 cells per 2.5 cm from Tosyl-BSA-immunized *rabbits* (*Edelman* et al., 1971; Table 2, experiment 2). The same fibers bound exactly the same number of cells from normal *mice* (130 cells per 2.5 cm) and Tosyl-BSA-immunized *mice* (297 cells per 2.5 cm) (*Rutishauser* et al., 1972, Table 1)! Mouse B- and T-lymphocytes (see above) bound to antigen-coupled fibers at 4° C (*Rutishauser* and *Edelman*, 1972). The binding was inhibited by the relevant antigen, if present in soluble form in the separation medium and by antimouse Ig but not by sodium azide (*Rutishauser* et al., 1972). Inhibition of binding to DNP-BSA fibers with BSA or DNP-lysine indicated that the fiber binding was specific mainly for the hapten and to a much lesser degree for BSA or new antigenic determinants created by the DNP-BSA conjugation. The number of binding cells and the binding avidity as determined by inhibition of binding in the presence of different concentrations of soluble antigen or hapten was higher in cell populations prepared from immunized animals than from normal animals. Approximately 1% of normal spleen cells and up to 17% of spleen cells from immunized mice bound to DNP-BSA fibers (*Rutishauser* et al., 1973). B and T cells in fetal liver and spleen bound to fibers coupled with a variety of antigens and haptens with the same relative avidity as adult cells (*D'Eustachio* and *Edelman*, 1975). The advantage of the fiber technique is that antigen-binding cells can be recovered in suspension. However, the functional properties of *normal* fiber-binding T and B cells have not yet been studied.

In contrast to fiber separation, hapten-gelatin layer-binding cells still have hapten-gelatin bound to the surface after recovery by melting (*Haas* et al., 1974; *Haas* and *Layton*, 1975). This material could be removed from the cells as monovalent hapten after treatment with collagenase. The capacity of purified collagenase-treated cells to rebind the appropriate hapten was shown by rosette formation with haptenated SRC (*Haas* and *Layton*, 1975) or immunofluorescence (*Nossal* and *Layton*, 1976). Up to 80% of NIP-gelatin-purified cells formed

rosettes with NIP-SRC, depending on the conjugation ratio of the gelatin and the SRC with the hapten (*Haas*, 1976, unpublished data). Cell suspensions containing 30%–40% readily detectable ABC after hapten-gelatin separation facilitated studies on specific antigen receptors of B-lymphocytes (*Nossal* and *Layton*, 1976; *Goding* and *Layton*, 1976).

Antibody-Forming Cell Precursors (AFCP) of Normal Animals

Wigzell and *Mäkelä* (1970) passed mixtures of spleen and lymph node cells through antigen-coated plastic bead columns and tested unfractionated, passed, and retained cells for their capacity to mount a specific antibody response in irradiated syngeneic recipients. The capacity to produce antibodies directed to a hapten (NIP) or proteins (HSA, BSA, OA) was reduced and in some cases completely abolished in the cell populations that had passed through the columns coated with the relevant hapten or antigen. The enrichment of specific bone marrow cells in such columns described by *Abdou* and *Richter* (1969) is difficult to understand since a large proportion of AFCP was retained nonspecifically. *Davie* and *Paul* (1970) and *Davie* et al. (1971) used a batch procedure to bind AFCP present in spleen and lymph nodes from normal guinea pigs to DNP-carrier-coupled agarose beads and obtained essentially the same data as *Wigzell* and *Mäkelä* (1970). Further specific cell separation experiments performed by *Wigzell* and his colleagues strongly suggest that the failure of the nonbinding cells to mount an adoptive humoral immune response was in fact due to the retention of AFCP in the columns and not to the retention of T helper cells (*Wigzell*, 1970, 1971; *Wigzell* et al., 1971; *Singhal* and *Wigzell*, 1970, 1971). Similarly, passage of spleen cells from mice, injected 1 h before with TNP-Fl-protein conjugates, through columns coupled with anti-Fl antibodies, resulted in partial depletion of the in vitro anti-TNP antibody response (*Scott*, 1976a).

The first impressive *enrichment* of AFCP was achieved by *Henry* et al. (1972). Transfer of thymocytes and only 6×10^3 normal spleen cells eluted from lactoside-coupled polyacrylamide bead columns in the presence of free lac hapten gave an antilac AFC response similar to the response obtained after transfer of 2×10^7 unfractionated cells.

More recently, enrichment of hapten-specific AFCP present in normal mice was also achieved consistently by the gelatin melting technique. Small numbers of unfractionated and hapten-purified cells were stimulated in microcultures by hapten conjugated onto a T-independent carrier protein in the presence of various types of filler cells (*Haas*, 1975; *Nossal* and *Layton*, 1976; *Stocker*, 1976; *Nossal* et al., 1976). Comparison of the cell dose-response curves revealed specific enrichment of AFCP in the purified cell populations. The degree of enrichment was mainly dependent on the hapten density of the adsorbent. More cells were bound with increasing hapten density of the gelatin layers. The additional binding cells using highly hapten-coupled gelatin had surface Ig with low affinity for the hapten and were not stimulated by soluble antigen. If B cell mitogens were used for stimulation, more low affinity receptor-bearing cells differentiated into AFC, which secreted low affinity antihapten antibodies as

detectable in a hemolytic plaque assay using highly coupled indicator red cells (*Haas*, 1976, unpublished data). Low hapten-coupled gelatin should be used in order to enrich AFCP bearing relative high affinity receptors. The frequency of specific antihapten AFCP in purified spleen cell populations was about 2×10^{-2} compared to $1\text{–}3 \times 10^{-4}$ in unfractionated spleen cells (*Nossal* and *Pike*, 1976). Even higher enrichment (3×10^{-1}NIP-specific AFCP) was obtained by two sequential separations on NIP-gelatin layers and a final purification step using a specific rosette fractionation procedure (*Nossal* et al., 1976; *Nossal* and *Pike*, 1978).

Memory B Cells

The first specific cell separation was performed by *Wigzell* and *Andersson* (1969) using antigen-coupled plastic or glass bead columns and cells from immunized animals. Cells that passed through the column were specifically depleted in memory B cells, as demonstrated in an adoptive transfer system. Further experiments suggested that the surface Ig of memory B cells had the same fine specificity, i.e., cross-reactivity (*Wigzell* and *Mäkelä*, 1970; *Rubin* and *Wigzell*, 1973), affinity for antigen (*Andersson*, 1972), and heavy chain (*Walters* and *Wigzell*, 1970; *Wigzell* and *Mäkelä*, 1970) as the Ig secreted by members of their progeny. However, the existence of a subset of memory cells with entirely different properties could not be excluded, since a substantial proportion of memory cells were retained nonspecifically in the bead columns (*Eckert* and *Pasternak*, 1973). The high rate of nonspecific retention disqualified such columns for attempts to enrich AFCP of a given specificity.

Some enrichment of anti-DNP AFCP from DNP-hemocyanin-primed mice was achieved by *Rutishauser* et al. (1973) using the nylon fiber fractionation technique. DNP-BSA-coupled or DNP-gelatin-coated fibers bound DNP-specific lymphocytes that were recovered by plucking or melting. Purified DNP-binding cells gave two to three times more anti-DNP IgG AFC as the same number of unfractionated cells 7 days after transfer together with helper T cells into irradiated syngeneic recipients. The IgM response of purified cells was not enriched. The presented data do not allow an estimation of the degree of enrichment of DNP-specific B memory cells in the fiber-binding cell population.

Waldmann (1977), using the gelatin dish separation technique, also failed to obtain more than a twofold to threefold enrichment of DNP-specific B memory cells. Possibly, this failure was due to an insufficient tendency of B memory cells (*Schrader*, 1974) or of a subpopulation of B cells (B2) to adhere (*Quintans* and *Cosenza*, 1976). Alternatively, it was a technical failure (insufficient stability of the gelatin layer or insufficient collagenase treatment after the separation).

Antibody-Forming Cells (AFC)

AFC are the only lymphocytes whose specific immunocompetence can be identified readily at the single cell level by hemolytic plaque techniques. They still

exhibit, at least in early differentiation stages, surface receptors for antigen and are therefore well-suited for the study of the relationship between surface Ig and secreted Ig at the level of the activated B cell. The following results were obtained with "immunoadherence" of AFC:

1. Antialbumin and antihapten antibody-secreting cells were retained in albumin- or hapten carrier-coated plastic bead columns. Antihapten AFC like antihapten antibodies did not recognize any determinants of the carrier to which the hapten was coupled. This was found with immune cells from mice (*Wigzell* and *Mäkelä*, 1970) and guinea pigs (*Davie* and *Paul*, 1970).

2. Low affinity anti-BSA AFC (*Wigzell*, 1970), anti-DNP AFC (*Andersson*, 1970), or antilactoside AFC (*Wofsy* et al., 1971) appeared to sneak through the columns coated with the appropriate antigens or haptens.

3. Surface Ig and secreted Ig appeared to belong to the same Ig class (*Walters* and *Wigzell*, 1970; *Wigzell* and *Mäkelä*, 1970). In all these studies except one (*Wofsy* et al., 1971), it is conceivable that the absence of plaque-forming cells in the effluent fractions was at least partly due to "blockade" of antibody secretion by "picked-up" column antigen (*Schrader* and *Nossal*, 1974).

Experiments reported by *Bellone* et al. (1974) reflect a more complex situation. These authors passed spleen cells of mice primed with arsanilic-KLH conjugated through hapten-conjugated polyacrylamide bead columns and found that the priming dose and the interval between priming and boosting were critical for antihapten AFC, depletion. However, the affinity of secreted antihapten antibodies did not account for the observed differences in depletion patterns of AFC.

Enrichment of anti-SRC AFC was achieved by *Mage* et al. (1969) who used a SRC-coated reticulum of an open pore polymer foam. The binding cells recovered by squeezing the foam contained six times as many anti-SRC AFC as did unfractionated cells. A much higher enrichment of antilactoside AFC was obtained by *Wofsy* et al. (1971) using a batch adsorption procedure. The AFC were recovered from lactoside-coupled polyacrylamide beads by elution with free lactoside. A similar procedure was used by *Tlaskalova* et al. (1975) who recovered lymphoid populations enriched in anti-HSA or anti-SRC (7S and 19S) plaque-forming cells from antigen-coupled spheron beads by mechanical means.

Greely et al. (1974) used red cell monolayers both as an adsorbent and as an indicator for AFC. The authors found that anti-TNP AFC adhered at 4° C as well as at higher temperatures to monolayers of TNP-coupled goat red cells with various hapten densities, whereas antigoat erythrocyte AFC bound specifically to goat red cell monolayers only at 25°–37° C. However, in the presence of EDTA-dextran, antigoat erythrocyte AFC also adhered at 4° C, indicating the influence of charge effects (*Greely* et al., 1974).

c) Separation of Specific T Cells

Antigen-Binding T Cells and T Cell Receptors (Nonfunctional Studies)

Nylon fibers coupled with haptens, proteins, or hapten-protein conjugates bound both splenic T and B cells but no thymocytes at 4° C (*Rutishauser* and *Edelman*,

1972). Although the total number of binding cells varied considerably, the proportion of T and B cells was similar in unfractionated and fiber-binding spleen cells from normal and immunized mice. The binding of both cell types to DNP-BSA fibers was inhibited by free hapten and anti-Ig. In contrast, *Kiefer* (1975) found that TNP-fiber binding of T-lymphocytes was inhibitable with free DNP but not with anti-Ig serum. Experiments described by *Krawinkel* and *Rajewsky* (1976) also suggest that T cells bind via hapten-specific receptors that do not react with anti-Ig sera directed against all known mouse Ig classes. The authors incubated splenic T- and B-lymphocytes from immunized mice with hapten or antigen-coupled nylon fibers for 1 h at 4° C. Nonbinding cells were then removed and the bound cells were released during a 2-h incubation period at room temperature. Antigen-binding material could be recovered from the fibers, the majority of which bound to anti-Ig-coupled immunoadsorbents. A minor fraction did not bind and further experiments strongly suggest that it represented hapten-specific T cell receptors. The anti-Ig-negative T cell receptor fraction carried the variable region of the Ig heavy chain (*Krawinkel* et al., 1976, 1977a) and – as demonstrated with hapten-binding receptors isolated from immunized rabbits – allotypic determinants of Ig heavy chains (*Krawinkel*, 1977b). Further studies are required to clarify the function of the antigen-binding T cells and their receptors described in this Section.

Helper and Suppressor T cells

Wigzell and his colleagues attempted to deplete carrier-specific helper T cells required for adoptive antihapten antibody responses by passage of various immune cell suspensions through carrier or hapten carrier-coated Degalan bead columns at 4° C (*Wigzell*, 1970; *Wigzell* et al., 1971) or Sepharose bead columns at room temperature (*Rubin* and *Wigzell*, 1973). T helper cells were never retained in such columns. This would not be surprising if the mechanism of specific cell trapping in columns is based on selective flow rate reduction and final nonspecific adherence (*Wigzell*, 1970) since T cells have much less tendency to adhere nonspecifically than B cells.

Maoz et al. (1976) reported that they achieved enrichment and depletion of antigen-specific helper cells using collagen-coated dishes coupled with KLH or (T,G)-A-L. Nylon wool purified T cells from in vitro or in vivo carrier-primed spleen cells were incubated in antigen-coated dishes on a rocker for 60 min at 37° C. Nonadherent cells were removed by two washes with medium, and the bound cells were recovered from the adsorbent by digesting the gel with collagenase. Adherent cells were enriched and nonadherent cells depleted in T cells capable of providing help to unprimed spleen cells in their in vitro antihapten response to hapten conjugated with the appropriate carriers. A similar specific fractionation effect was demonstrated for in vitro induced suppressor T cells. The low efficiency of the culture system used by *Maoz* and his collaborators constitutes a serious objection to this data.

Rubin (1976) found enrichment of helper activity in immune mouse spleen cells after passage through Ig-anti-Ig glass bead columns or antigen-coated polyacrylamide bead columns. The results were interpreted to mean that the

columns retained regulatory (suppressor) T cells that could not be recovered from the adsorbents.

Clear enrichment of antigen-specific suppressor T cells was recently reported by two laboratories. *Okumura* et al. (1977) used mice primed with soluble KLH or KLH and DNP-KLH in alum with pertussis vaccine as a source of KLH-specific suppressor T cells or helper T cells and DNP-specific B cells. KLH-immune cells were passed through antimouse Ig-coupled Sephadex 200 bead columns at 4° C to deplete B cells and then through the same columns coated with KLH at 37° C. The cells were left in the columns for 30 min before nonbinding cells were removed by washing with warm (37° C) medium. Adherent cells were then removed by washing with cold medium at a flow rate of 1 ml/min. Approximately 0.5% of the original cells from KLH-immune cells were recovered but less than 0.1% from egg albumin-immune cells. A small number of specifically adherent cells (2×10^5) suppressed a secondary anti-DNP response to DNP-KLH in vitro or in vivo completely while 10^7 nonbinding cells showed no suppression. Helper cells from KLH (in alum with pertussis) primed mice were not retained in KLH columns, in contrast to a small number of KLH-specific suppressor cells also present in these mice. The purified suppressor cells were characterized as I-J$^+$, Ly2,3$^+$ and FcR$^-$ T cells and after sonication provided a good source of antigen-specific suppressor factors.

An even simpler technique was described by *Taniguchi* and *Miller* (1977) who enriched T cells in plastic dishes coated with antimouse Ig and then HGG-specific suppressor cells in dishes coated with HGG. Specific suppressor cells bound during a 1-h incubation period at room temperature and could be recovered after removal of nonbinding cells from the plates kept on ice for 15 min. The purified population was highly enriched for HGG-specific cells suppressing an adoptive transfer response to DNP-HGG. They also expressed Ly2,3$^+$ and I-J$^+$ surface markers.

Proliferative T Cell Response to Conventional Antigens

T cells from immunized animals respond to antigen in vitro with an increase in DNA synthesis and proliferation. The in vitro proliferative response to a hapten carrier conjugate is—like delayed hypersensitivity reactions in vivo—highly specific for the immunizing conjugate. *Davie* and *Paul* (1970) found that "absorption" of cells from DNP-GPA-immunized guinea pigs with Sepharose beads coupled with DNP-GPA depleted T cells that proliferated in vitro upon stimulation with DNP-GPA. DNP-GPA responder cells were, however, not depleted with Sepharose beads coupled with DNP-BSA, DNP-lysine, or GPA conjugated with DNP-related haptens. The authors suggest that the T cells recognized, unlike B cells and their Ig products, the hapten as well as part of the carrier. *Rubin* and *Wigzell* (1974), using almost identical experimental conditions, failed to confirm these data.

Webb et al. (1975) fractionated lymph node cells from guinea pigs sensitized to basic encephalitogen (BE) using BE-coupled collagen or gelatin layers. The in vitro response to BE, as measured by ^{3}H-thymidine incorporation, was slightly

enriched in the binding cells and reduced in the nonbinding cells. It is not clear whether ^{3}H-thymidine was incorporated only by T cells.

Cytotoxic T-Lymphocytes (CTL)

More recently, three groups reported their attempts to separate cytotoxic T cells using target cell antigens rather than whole cells.

1. *Maoz* and *Shellam* (1976) separated cytotoxic T cells from tumor-immune rats on collagen gels coupled with tumor cell membrane extracts (TAA). Cells which did not bind at 37° C were depleted in cytotoxic activity. However, in contrast to the interpretation of the authors, the data show no enrichment of TAA-specific CTL in the adherent population that was recovered after collagenase digestion of the gels (*Maoz* and *Shellam,* 1976).

2. *Bröcker* and *Sorg* (1977) described a new immunoadsorbent that was prepared by polymerizing a mixture of gelatin and polyethyleneimine with glutaraldehyde on the bottom of a Petri dish. Detergent-solubilized H-2 antigens were conjugated covalently to unsaturated aldehyde groups of the film. Nylon wool column purified spleen cells from alloimmunized mice were incubated on the films. The action of cytotoxic T cells in unfractionated cells, nonbinding cells, and binding cells recovered by mechanical means was then tested in a microcytotoxicity assay. High enrichment and depletion of specific T cells was achieved.

3. *Scott* (1976a) failed to retain in anti-Fl columns the precursors of cytotoxic T cells for TNP-modified syngeneic cells after exposure in vitro to TNP-Fl-protein. Some allogeneic killer T cells (*Scott,* 1976a; *Singer* et al., 1978) but not their precursors (*Scott*, 1976a) were retained after exposure to Fl-coupled *target cells* in anti-Fl columns. All these studies are somewhat preliminary and need to be confirmed and extended.

Graft Versus Host (GvH) Reactive Cells

Haskova et al., (1974) passed lymphocytes from normal Lewis rats or from Lewis rats grafted with allogeneic (AVN) skin through columns containing Spheron beads coupled with AVN or Lewis transplantation antigens. The passed cells and the mechanically recovered binding cells were tested in Fl hybrids for GvH reactivity in the popliteal lymph node weight assay. No significant differences between experimental and control groups were found, indicating that specific GvH reactive cells did not bind to the immobilized preparations of transplantation antigens.

Binz and *Wigzell* (1975c), using antireceptor antibodies, were able to separate *specific* GvH reactive rat T-lymphocytes. Purified T cells were incubated with anti-idiotypic antibodies and then passed through Degalan bead columns coated with Ig-anti-Ig. The passed cells were specifically depleted in GvH reactivity to the appropriate cells and the binding cells showed reactivity only in the appropriate Fl hybrids. In addition, the specificity of the separation was demonstrated by using two different types of anti-idiotypic antibodies.

D. Cell Monolayers Used as Immunoadsorbents

Brondz and his colleagues (*Brondz*, 1968; *Brondz* and *Golberg*, 1970; *Brondz* and *Snegiröva*, 1971) were the first to demonstrate that sensitized lymphoid cell populations could be depleted of their cytotoxic activity by "absorption" on monolayers of the sensitizing cell type. Since the killing of target cells was shown to require direct contact with the killer cells (*Wilson*, 1965; *Rosenau*, 1968; *Cerottini* and *Brunner*, 1974; *Golstein* and *Smith*, 1977; *Henney*, 1977; *Martz*, 1977), the depletion of cytotoxic activity in monolayer-absorbed cell populations is usually interpreted to indicate that the cytotoxic T cells are retained on the immobilized monolayer cells. Monolayer separation techniques were applied in many attempts to separate various types of antigen-specific T cells. From the following description, however, the reader may realize that these cell separation techniques, at least at the present stage, are still of rather limited value in immunology.

1. Preparation of Monolayers

Macrophage monolayers were used by *Brondz* and his colleagues (*Brondz*, 1968; *Brondz* and *Snegiröva*, 1971; *Brondz*, 1972; *Brondz* et al., 1975a, b). Peritoneal exudate cells (PEC) were obtained 2–3 days after i.p. injection of an irritant and cultured in flat-faced tubes or plastic bottles to obtain almost confluent monolayers. Adherent antigen-pulsed macrophages were used by *Ben-Sasson* et al. (1975a) and by *Swierkosz* et al. (1978) in attempts to separate T cells with specificity for non-MHC antigens (Sect. IV.C).

Preparation of *fibroblast monolayers* has been described by *Ginsburg* (1968) and *Berke* et al. (1969). The fibroblasts were obtained by trypsinization of embryos or embryonic skin (*Rubin*, 1975) and cultured in plastic Petri dishes. The cultured cells were trypsinized in order to detach them, and "secondary cultures" were incubated until confluent monolayers were grown. *Adherent tumor cells* (*Kedar* et al., 1974) or peripheral blood cells (*Bach* et al., 1973) were also used for cell separation.

A new technique was introduced by *Stulting* and *Berke* (1973a) who prepared cell monolayers by electrostatic binding of cells to plastic dishes treated with poly-L-lysine (PLL) (*PLL monolayers*). This technique allows the preparation of monolayers with any cell type, like red cells (*Kennedy* and *Axelrad*, 1971), tumor cells (*Stulting* and *Berke*, 1973a), or normal spleen cells (*Bonavida* and *Kedar*, 1974; *Kedar* and *Bonavida*, 1975).

Kiefer (1978) developed a technique that allows the creation of diazonium groups on the surface of polystyrene plastic dishes. Any cell type may be radily coupled *covalently* to such dishes in cold temperatures at physiologic pH. No cells are detached from the plates if they are kept for several hours at 4° C, but a few cells detach during a 2-h rocking period at 37°C (*Haas*, 1976, unpublished data).

Monolayers may be irradiated if necessary (*Feldman* et al., 1972; *Ben-Sasson* et al., 1975a). Glutaraldehyde-fixed fibroblast monolayers could be used for binding of cytotoxic T cells (*Golstein* et al., 1972) and their precursors (*Wekerle*

et al., 1974), while glutaraldehyde-fixed macrophage monolayers apparently lost their ability to bind specific killer cells (*Brondz* et al., 1975b). Tumor cell monolayers fixed with formaldehyde in low concentrations were used for cytotoxic T cell binding by *Stulting* et al. (1975).

2. Lymphocyte Separation

a) Contact Between Cells to be Separated and Monolayers

The cells to be separated are added to the monolayers in a concentration of about 10^7 cells per milliliter medium and the plates are then incubated without rocking or with occasional or continuous agitation for 1–4 h (Table 3) or even for 10 h (*Clark* and *Kimura*, 1972). The interaction of T cells with other cells appears to require metabolic energy. Thus, adsorption of killer cells (*Golstein* et al., 1971) and their precursors (*Wekerle* et al., 1972) to monolayers occurred at 37° C but not at 4° C. It has been suggested that the energy is required to expose partly hidden receptors because killer cell precursors adhered to monolayers at 4° C if pretreated with neuraminidase (*Wekerle* et al., 1972).

Often more than one separation is required for complete depletion of specific T cells. *Kedar* et al. (1974) introduced a new technique in which the adsorption process is accelerated by a 5-min centrifugation period. Ten to twenty million spleen cells in 2 ml medium are added to various monolayers in 60-mm tissue culture dishes that are then incubated for 15 min at 37° C with or without rocking. The plates are then centrifuged at 80 *g* for 5 min at 24° C in a swinging bucket rotor. Nonadherent cells are collected with two washings or separated once more on a fresh monolayer. This technique appears to allow a more efficient depletion of cytotoxic T cells as compared to a 3-h incubation technique (*Kedar* et al., 1974). It has been argued that T cell receptors may be imbedded in the cell surface and/or shielded by charged groups and that centrifugation might be to overcome these obstacles to binding (*Mage* and *McHugh*, 1975).

b) Recovery of Nonadherent Cells

Nonadherent cells are usually poured off the plates or aspirated with or without previous mechanical agitation. It is conceivable that in most cases a substantial proportion of nonadherent cells were not removed from cell monolayers by mechanical agitation and two washes. Some authors tried to standardize the procedure for recovery of nonadherent cells (*Golstein* et al., 1971). Unfortunately, quantitative data were not presented on the recovery of nonadherent cells and their "contamination" by detached monolayer cells after various degrees of mechanical agitation. However, functional tests revealed no difference in the depletion of specific T cells regardless of the degree of agitation employed (*Golstein* et al., 1971), indicating that specifically bound cells could not be removed from the monolayers by mechanical means. Alternatively, vigorous agitation removed killer cells and monolayer cells in a sufficient number to effect cold target inhibition.

Table 3. Monolayer Separation of Specific T-Lymphocytes

Cells separated	Monolayers	Separation	Result	References
Lymph node	Macrophages	Incub. (2× up to 6 h)	CTL (d+)	*Brondz* and *Snegiröva* (1971); *Brondz* et al. (1973a, b)
Spleen	Fibroblasts	Incub. (1×3–4 h)	CTL (d+, e±)	*Goldstein* et al. (1971, 1972, 1973); *Berke* and *Levy* (1972)
PEL	Tumor cells (PLL)	Incub. (1×40 min)	CTL (d+)	*Stulting* and *Berke* (1973a, b); *Stulting* et al. (1975)
Spleen	Fibroblast, tumor cells, spleen (PLL)	Incub. (1×3 h) or 2–3×15 min + centr.	CTL (d+)	*Kedar* et al. (1974); *Kedar* and *Bonavida* (1975)
Spleen	Fibroblasts	Incub. (2–3×1 h)	CTL (d+)	*Henney* and *Bubbers* (1973)
Spleen	Tumor cells (PLL)	Incub. (2×40 min)	CTL (d+) i CTLP (d+) pre-CTL (d−)	*Kamat* and *Henney* (1975)
Spleen	Macrophages	Incub. (1×4 or 2×2 h)	CTL (d+, e+)	*Brondz* et al. (1975a, b)
Spleen	Spleen (PLL)	Incub. (1×45 min) + centr. (5 min)	CTL (d+) n+i CTLP (d−)	*Neefe* and *Sachs* (1976, 1977)
Spleen	Tumor cells (PLL)	Incub. (2×40 min)	Con A-med. cytolysis (d−)	*Rubens* and *Henney* (1977)
Lymph node	Fibroblasts[a]	Incub. (1×0,5–6 h)	CTLP (d+)	*Lonai* et al. (1972); *Clark* and *Kimura* (1972); *Wekerle* et al. (1972, 1973a, b, 1974); *Altman* et al. (1973); *Wekerle* and *Feldman* (1974)
Thymus[a] Lymph node[a]	Fibroblasts[a] Thymus reticulum[a]	Incub. (1×1–5 h)	Recruiter T cell (d+, e±)	*Cohen* and *Wekerle* (1973a, b, 1974)
Lymph node, spleen	Fibroblasts	Incub. (1×2–18 h) or shaking (2 h)	CTLP (d+) GvHR (d+)	*Lonai* et al. (1973a, b)
PBL	Adh. PBL	Incub. (1×1 h)	CTL, CTLP (d+) MLR (d−)	*Bach* et al. (1973)
Spleen	Spleen (PLL)	Incub. (1×45 min) + centr. (5 min)	CTLP (d+) GvHR (d+)	*Bonavida* and *Kedar* (1974)

Table 3 (continued)

Spleen	Fibroblasts, macrophages	Incub. (1 × 3 h)	CTL (d+) GvHR (d−)	*Mage* and *McHugh* (1973)
Spleen	Spleen (PLL)	Incub. (2–3 × 0.5–1 h) + centr. (5 min)	CTL (d+) GvHR (d+)	*Mage* and *McHugh* (1975)
Spleen, thymus, lymph node	Fibroblasts	Incub. (1 × 3 h)	GvHR (d−)	RUBIN (1975)
Spleen	Macrophages	Incub (1 × 1 h)	pr. MLR (d−) sec. MLR (d+)	*Gorczynski* and *Rittenberg* (1975)
Spleen	Fibroblasts	Incub. (1 × 3 h) + shaking (90 min)	spec. B-cells (d+) GvHR (d+, e+)	*Binz* et al. (1974a); *Binz* et al. (1974b)

Depletion of specific cells: d+; no depletion: d−; enrichment of specific cells: e+; n: normal; i: immune.
[a] Cells were from rats, all other cells were from mice.

Release of Adherent Cells by Mechanical Means

Experiments performed by *Golstein* et al. (1971) and *Martz* (1975) suggest that the binding forces attaching cytotoxic T cells to the appropriate target cells are stronger than shearing forces generated by vigorous mechanical agitation. Therefore, other techniques were required to separate cells that adhere to each other.

c) Recovery of Adherent Cells

Most of the investigators using monolayers as a means to separate specific T cells did not intend to *isolate* specific lymphocytes. Their experiments aimed at demonstrating the depletion of a specific precursor or effector cell function in the nonadherent cell population. However, the lack of a certain specific function in the nonadhering lymphocytes does not prove the absence of cells with the appropriate specificity. The reduction or absence of the function under study may be due to inactivation or inhibition of specific cells by monolayer cells or soluble products of monolayer cells or by detached monolayer cells. Therefore, it is essential to recover the specific adherent cells and to demonstrate their function. Various methods that were more or less successfully employed to recover adherent cells are briefly described in the following.

Spontaneous Release

The adherence of specific cytotoxic T cells to appropriate monolayers may be demonstrated directly by a cytotoxic effect on the monolayer cells (*Brondz*,

1968, 1972). In the case of precursor cells, the monolayer may be used for adherence as well as for sensitization of specific cells. After 48 h the specific adherent cells start to detach from the monolayer and differentiate to mature cytotoxic T cells that can be tested on fresh monolayers (*Feldman* et al., 1972). Experiments by *Martz* (1975) indicate that although adhesions of CTL to target cells do not form at 0° C, once formed at higher temperatures, they did not dissociate spontaneously at 0° C.

Trypsinization

Attempts to selectively elute adhering cells only by brief incubation with trypsin were unsuccessful (*Golstein* et al., 1971). It was necessary to trypsinize the monolayer with adhering cells for 30 min at 37° C. The trypsin-eluted cell population consisted of lymphocytes and fibroblasts that could be separated from each other by density gradient centrifugation (*Golstein* et al., 1971; *Berke* and *Levey*, 1972). The interphase of a discontinuous BSA gradient contained mainly fibroblasts while lymphocytes and red cells were in the bottom of the gradient. However, there was still a cross-contamination of both fractions of up to 7%. A further disadvantage of this technique is that trypsin abolishes the cytotoxic activity of killer cells (*Mauel* et al., 1970; *Brondz* et al., 1973b; *Todd*, 1975). Cytotoxicity reappears with time after the removal of the enzyme (*Berke* et al., 1972).

Pronase Treatment (Brondz et al., 1975b)

Macrophage monolayers with adherent lymphocytes were washed to remove serum and incubated for 30 min at 37° C with pronase. The monolayers were then rocked for 5 min, and detached lymphocytes were harvested. A second fraction of eluted cells was obtained after further pronase treatment for 10 min. The eluted cells were pooled and the pronase activity "neutralized" by excess of bovine serum. Pronase treatment did not cause detachment of monolayer cells. The cytotoxic activity of eluted cells was abolished initially but reappeared in the course of a cytotoxicity assay lasting 16–20 h.

Release in the Absence of Divalent Cations

Stulting and *Berke* (1973a, b) showed that CTL could be recovered from monolayers in the presence of EDTA, and *Martz* (1975) found that 10-min incubation with EDTA followed by vigorous vortex shaking for 10 s resulted in complete disruption of adhesive bonds between CTL and target cells. Other authors, however, found that the monolayers, even though stabilized by poly-L-lysine, were destroyed by this procedure (*Brondz* et al., 1975b; *Neefe* and *Sachs*, 1976). Moreover, *Golstein* et al. (1971) failed to elute adherent killer cells using EDTA, a finding consistent with the data of *Henney* (1973), suggesting that EDTA does not inhibit lymphocyte target cell binding. For a more detailed discussion of the effects of Mg^{2+} and Ca^{2+} on killer cell target cell interactions, see *Henney* (1977) and *Martz* (1977).

Release at Low pH Values

Stulting and *Berke* (1973a) also tried to elute adherent killer cells from monolayers at low pH values since deviation from physiologic pH values was shown to interfere with the cytolytic reaction. Indeed, functional intact killer cells appeared to detach from the target monolayers at pH 5.

3. Applications

a) Number of Monolayer Binding Cells

It was observed a long time ago that sensitized lymphocytes tended to adhere in greater numbers to the relevant target cells than to others (*Koprowski* and *Fernandes*, 1962; *Rosenau*, 1963; *Wilson*, 1965). *Lonai* et al. (1973a) found that about 4% of ^{51}Cr-labeled normal Balb/c lymph node cells bound to allogeneic fibroblast monolayers. The fraction of adhering cells dropped to a quarter of that of the first adherence step if the nonbinding cells were separated again on the same allogeneic monolayer, but not if separated again on a third party monolayer. Only about 1% of ^{51}Cr-labeled cells bound to syngeneic monolayers. Similarly, 2%–5% of rat lymphocytes were attached to xenogeneic monolayers such as mouse fibroblasts even after a 10-h incubation period (*Clark* and *Kimura*, 1972). Subsequently, such low rates of lymphocyte binding to monolayers were not found by other authors. In many cases, considerable nonspecific binding was observed, 25%–75% of the original cell input adhered to the monolayers irrespective of the H-2 type of the adsorbing monolayer cells (*Golstein* et al., 1971; *Brondz* et al., 1975b; *Neefe* and *Sachs*, 1976). The considerable differences between the binding rates obtained in different laboratories is probably due to differences in the cell population to be separated, different properties of the monolayers, different separation procedures (rocking or no agitation), and differences in the procedures employed to recover nonadherent cells. In many cases somewhat lower numbers of binding cells were obtained with more extensive washing of the monolayers after the incubation. However, it is quite clear that monolayer separation techniques at the present stage do not allow quantitation or purification of alloreactive T cells.

b) Cytotoxic T-Lymphocytes (CTL) and Their Precursors (CTLP)

Depletion of cytotoxic T cells using cell monolayer separation techniques has been reported by many investigators (Table 3). In all cases cytotoxic activity was reduced, if not completely abolished, in the nonadherent cell populations. The observed depletion of cytotoxic activity was thought to be due to specific binding of killer cells to target cell monolayers. However, one or more of the following objections can be made, some of which were discussed elsewhere by *Neefe* and *Sachs* (1976):

1. Cytotoxicity was not shown to be T cell mediated.
2. The specificity of depletion was not shown (criss-cross absorptions using two types of immune cells and monolayers).

3. Lytic activity was not quantitated in the unfractionated and nonadherent cell populations by titration of effector cells.

4. It was not excluded that cytotoxic T cells were present in the nonadherent population, the killing activity of which was not detected because of "contaminating" monolayer cells (cold target inhibition) or blockage by antigens picked up from monolayer cells during the separation period.

Only a few studies suggest strongly that the depletion of cytotoxicity was indeed, at least partially, due to specific depletion of cytotoxic T cells (*Golstein* et al., 1971; *Brondz* et al., 1975b; *Neefe* and *Sachs*, 1976, 1977). For many purposes, alternative techniques may be used: cold target inhibition for studies on the specificity of cytotoxic T cells (*Doherty* and *Zinkernagel*, 1974; *Bevan*, 1975; and PHA or Con A mediated killing (*Möller*, 1965; *Bevan* and *Cohn*, 1975) to distinguish requirements for binding and killing, although it has been argued recently that Con A mediates target cell lysis not only by binding killer to target cells (*Rubens* and *Henney*, 1977).

The demonstration of *enrichment of specific cytotoxic T cells* in the adherent cell population is essential to prove unequivocally the specific binding of cytotoxic T cells to monolayers. So far, specific killer cell enrichment in recovered monolayer adherent cells as compared to unfractionated cells has been demonstrated convincingly only in one study (about tenfold enrichment; *Brondz* et al., 1975b). For enrichment of cytotoxic T cells, alternative techniques appear to be more promising such as long-term "selection cultures" (Chap. IV).

So far it is not clear whether *the precursors* of cytotoxic T cells can be separated using monolayer techniques. Some authors were able to bind precursor cells specifically to monolayers (for review, see *Feldman* et al., 1972). However, more recent studies by *Neefe* and *Sachs* (1976) indicate that CTL precursors from normal and immunized mice failed to adhere specifically to immobilized target cells. This data, however, should not be taken to indicate that a close contact between responder and stimulator cells is not required or that CTL precursors are not precommitted to recognize a particular antigen. The results are more likely to be due to technical difficulties of depleting specific receptor bearing cells and to the lack of quantitative data on CTL precursor cells. Altogether it is conceivable that monolayer separation techniques can hardly be improved to allow a high degree of purification of CTL or their precursor cells.

c) Proliferative T Cell Response to Alloantigens

A large number of T cells appear to be able to respond to certain alloantigens expressed at the cell surface and coded for by genes in the major histocompatibility complex (MHC). Thus, the injection of lymphoid cells into immature or immunosuppressed allogeneic or F1 hybrid mice produces a GvH reaction that is often measured by determining the degree of splenomegalie or local lymph node enlargement. An in vitro correlate of this reaction is the T cell proliferative response in mixed leukocyte cultures (MLC) as determined by ^{3}H-thymidine incorporation.

Attempts to deplete GvH or MLC reactive T cells in vitro using monolayer separations have had variable results (Table 3). As the stimulation of T cells by allogeneic cells involves at least transiently cell-to-cell contact, it is conceivable that specific cell separation should be achieved at least to a certain degree, since the responding cells probably have restricted specificities (i.e., are precommitted; Sect. III and IV). However, further analysis of cell-cell interactions involved in GvH reaction (*Cantor* and *Asofsky*, 1972) and more quantitative assays for GvH-reactive cells are required to obtain more consistent results.

d) Separation of DTH T Cells and Helper T Cells for Humoral Immune Responses

Antigen-pulsed macrophages were recently used in attempts to separate specific DTH T cells (*Ben-Sasson* et al., 1975a) or T helper cells for humoral immune responses (*Swierkosz* et al., 1978). In both cases, the enrichment of specific T cells obtained was probably mainly due to preferential stimulation (positive selection) rather than to separation of reactive T cells by the antigen-pulsed macrophages. Therefore, these techniques are described in Section IV.C.

E. Rosette Separation Techniques

The adherence of large particulate antigens such as bacteria (*Hayes* et al., 1951; *Mäkelä* and *Nossal*, 1961; *Russel* and *Diener*, 1970), antigen-coated bentonite beads (*Baker* et al., 1966), or red cells (*Nota* et al., 1964; *Zaalberg*, 1964) has primarily been used to visualize specific AFC. The binding of red cells to lymphocytes (rosette formation) has since been studied in great detail and is now used to also identify precursors of antibody-secreting lymphocytes bearing M-Ig of a particular specificity as well as nonspecific lymphocyte subpopulations. The advantage of rosette techniques is that the rosetting cells can be separated from nonrosetting cells on the basis of considerable size and density differences (Table 4).

1. Indicator Red Cells and Specificity of Rosette-Forming Cells (RFC)

Heterologous erythrocytes were used for immunocytoadherence in most studies on enrichment and depletion of RFC. In these cases, RFC are rather heterogeneous with regard to M-Ig specificities, since red cells have a variety of different surface antigens. In principle, the rosette technique is applicable to a variety of different antigens and haptens which can be coupled to the erythrocyte surface. However, it is still difficult to obtain indicator red cells that bind only to lymphocytes of a defined specificity. The coupling procedures can generate new antigenic determinants that are not present on the red cells and the coupled material. Coupling of large amounts of foreign material causes a decrease in original red cell determinants exposed at the red cell surface; however, if the epitope density of a particular coupled determinant is high on the erythrocyte surface, even cells bearing very low affinity receptors for this determinant

Table 4. Separation of specific T an B rosette-forming cells

Source of lymphocytes	Red cells	Rosette separation	RFC include	References
Spleen	SRC, PRC	BSA gradient	AFCP	*Brody* (1970); *Brody* and *Papermaster* (1970)
Bone marrow	SRC, PRC		AFCP	
Peyers-P.	SRC			
Spleen	SRC, CRC	Ficoll gradient	AFCP	*Bach* et al. (1970)
Spleen	SRC, HRC	Ficoll gradient	AFCP	*Gorczynski* et al. (1971)
Spleen	SRC	Velocity sed.	AFCP no T helper cells	*Osoba* (1970); *Haskill* and *Marbrook* (1971); *Elliott* et al. (1973)
Spleen	SRC	BSA gradient	AFC	*Maov* and *Harris* (1970)
Spleen	SRC	Velocity sed.	AFC	*Wilson* (1971); *Ambrosius* and *Steckel* (1972); *Lubbe* et al. (1976)
Spleen	SRC, DRC	Velocity sed.	B-MC (T helper cells)	*Wilson* (1973)
Spleen	HSA-SRC	Ficoll gradient	B-MC	*Sulitzeanu* and *Axelrad* (1973)
Spleen	SRC	Velocity sed.	B-MC no T helper cells	*Elliott* and *Haskill* (1973, 1975)
Spleen	FGG-SRC, DRC	Velocity sed.	B-MC	*Lafleur* and *Mitchell* (1975)
PBL[a]	SRC	BSA gradient	No GvH T cells	*Crone* et al. (1970)
Spleen	FGG-SRC, DRC	Velocity sed.	T helper cells	*Kontiainen* and *Andersson* (1975)
Lymph node	SRC	Velocity sed.	DTH T cells CTL	*Elliott* et al. (1975)

will form rosettes (*Möller*, 1974). A high degree of restriction in the specificity of RFC can be achieved by using autologous red cells coupled with anti-idiotypic Fab fragments (*Owen* et al., 1977).

Erythrocytes "sensitized" with antibodies or antibodies and complement or coupled with anti-Ig antibodies form rosettes with cells bearing Fc receptors, C3 receptors, or M-Ig respectively. Separation of antigen nonspecific RFC can serve as a model for specific rosette separation. Moreover, a rather indirect specific B cell separation has been described recently that is based on depleting specific cells of M-Ig by interaction with antigen (capping and shedding of M-Ig) followed by removal of M-Ig-dependent rosettes (*Walker* et al., 1977).

2. Rosette Formation Procedures

In the original rosette or cluster technique described by *Nota* et al. (1964) and *Zaalberg* (1964), lymphocytes are mixed with heterologous red cells. The suspension is kept for 1 h and then agitated gently to resuspend sedimented cells and newly formed rosettes (incubation technique). *McConnell* et al. (1969) and

Edwards et al. (1970) first described a modification of the incubation technique that involves acceleration of rosette formation by centrifuging the mixture of red cells and lymphocytes (centrifugation technique). The pellet is then gently resuspended after brief incubation. This technique appears to give a better yield of rosettes (*Gorzynski* et al., 1971) and has been used in recent years by most investigators with many slight modifications. Obviously in all rosette techniques the indicator red cells have to be in excess. Ratios of nucleated cells to red cells of 5:1 (*Wilson*, 1971; *McConnell* et al., 1969) to approximately 50:1 (*Kontiainen* and *Andersson*, 1975; *Brody*, 1970) were used. A ratio of 10:1 was found to be optimal by *Elliott* (1978). Simple balanced salt solutions are sufficient to allow rosette formation. However, it is important to keep the osmolarity and the pH constant to avoid changes in the erythrocyte volumes (see below).

In most cases, the temperature is kept at 4° C throughout the entire procedure of rosette preparation and separation. At this temperature, single layer rosettes are formed by direct binding of red cells to the lymphocyte surface via M-Ig specific for indicator cell determinants (for review, see *Warner*, 1974). At 37° C, large rosettes with several layers of red cells (clusters) are formed by AFC. RFC with single layers of red cells cap and shed the red cells at this temperature (*Ashman*, 1973; *Ashman* and *Raff*, 1973). Most investigators who attempted to obtain rosette formation by T cells kept the lymphocyte red cell mixture at least for a short period of time at room temperature or at 37° C. Rosettes should be counted by hemocytometer with crystal violet (*Elliott*, 1978) or acridine orange (*Kalland*, 1977) to insure the presence of a nucleated cell in the center of a rosette.

3. Stability of Rosettes

Some rosettes may dissociate during the handling of the suspension, which generates shearing forces between lymphocytes and red cells. The stability of rosettes is difficult to evaluate but an important parameter when rosettes are to be separated. Usually the resistance of rosettes to mechanical agitation is determined in a semiquantitative fashion. *Wilson* (1971) found that the stability of rosettes increases with time after immunization and suggested that this was due to the "maturation of the immune response," i.e., the relative increase of high affinity receptor-bearing cells. *Lafleur* and *Mitchell* (1975) believe that the rosettes most resistant to agitation are formed by lymphocytes with the highest density of M-Ig at the cell surface. *Haskill* and collaborators found that the most fragile rosettes are formed by antigen-specific T cells (*Haskill* et al., 1972; *Elliott* and *Haskill*, 1973), which were claimed by others to have a rather low density of cell surface receptors for antigen (for review, see *Roelants*, 1972). T-RFC bound less SRC than B-RFC and dissociated within 5 min at 4° C even with the most careful handling. The dissociation could be prevented by tratment of the rosettes with glutaraldehyde or by addition of sodium azide immediately after rosette formation (*Haskill* et al., 1972; *Elliott* and *Haskill*, 1973).

4. Separation of Rosettes

a) Velocity Sedimentation

Peterson and *Evans* (1967) and *Miller* and *Phillips* (1969) introduced a simple cell separation technique that is based on zonal sedimentation of cells at unit gravity (velocity sedimentation). The sedimentation velocity of a cell is determined by its size and buoyant density. Therefore, rosettes can be purified in a relatively short period of time since they differ considerably in size and density from single lymphocytes. Briefly, the cells are placed into the cone base of a separation chamber and suspension medium containing a shallow density gradient is pumped underneath lifting the cell suspension until it forms a thin band at the top of the cylindric region of the chamber. The cells are then allowed to sediment for several hours, and fractions containing cells that have sedimented according to size and density are collected via the bottom or top of the chamber. The velocity sedimentation of rosettes has been described in detail (*Edwards* et al., 1970; *Miller*, 1973; *Elliott*, 1978). The separation of rosettes is best accomplished in a flat separation chamber (*Miller*, 1973). The highest degree of rosette enrichment is obtained at the bottom fraction of the gradient, where 70% of the objects (cells, rosettes, cell aggregates, and clumps) were rosettes (*Edwards* et al., 1970). The broad distribution of rosettes over the whole chamber mainly reflects variations in the size of the rosettes. Some RFC bind many red cells and sediment quickly, other RFC bind fewer red cells or loose some red cells during the separation and sediment more slowly (*Edwards* et al., 1970; *Elliott* and *Haskill*, 1973). The enrichment of rosettes is decreasing with decreasing sedimentation velocity of rosettes. Complete depletion of RFC binding many red cells is obtained only in the upper fractions, which, however, still contain RFC binding fewer red cells (*Edwards* et al., 1970). The separation of various types of rosettes can be improved considerably by using prefractionated small lymphocytes for rosette formation (*Elliott* and *Haskill*, 1973).

b) Density Gradient Separation

Gradient centrifugation techniques have been used for a long time to separate cells according to differences in their buoyant density (for review, see *Shortman*, 1974). Rosettes can also be separated from single lymphocytes by centrifugation in density media since erythrocytes are more dense than lymphocytes under appropriate conditions.

Work of *Maov* and *Harris* (1968) using gradients of bovine serum albumin showed that lymphocytes having SRC bound to their surface banded at a density close to that of SRC alone. *Gorczynski* et al. (1971) used Ficoll density gradients for separation of rosettes. SRC banded in Ficoll at a density of 1080 g/cm^3 distinct from the lymphocytes capable of forming rosettes (density 1062–1070 g/cm^3). If rosettes were formed prior to separation, RFC with the red cells bound were shifted to the density interval near 1080 g/cm^3. Once the density of rosettes was defined, simplified density cut procedures could be used for RFC separation.

A major problem with macromolecular gradient media is their tendency to cause cell aggregations. Ficoll induces more aggregation than albumin but both induce aggregation at neutral pH (*Shortman*, 1972). With BSA the problem of cell clumping was overcome by lowering the pH to around 5.1 (*Shortman*, 1972). Both the addition of a dispersing agent and low pH were required to reduce aggregation in Ficoll (*Shortman*, 1972; *Gorczynski* et al., 1970). Low pH did not affect the volume or viability of nucleated cells but it caused marked swelling of red cells and a decrease in density (*Williams* and *Shortman*, 1972). Both effects are not desirable for rosette separation since the loss of red cell density decreases the density differences between rosettes and lymphocytes and swelling of red cells may cause dissociation of rosettes. Therefore, neutralized density media have to be used for rosette separation at the expense of more "contamination" of the rosette-enriched fractions with cell aggregates.

5. Applications

a) Separation of B Rosette-Forming Cells (B-RFC)

α) Antibody-Forming Cell Precursors (AFCP) in Normal and Immunized Animals

Normal mouse spleen cells depleted in SRC-RFC had a reduced capacity to generate anti-SRC AFC in irradiated adoptive recipients as compared to the unfractionated cell population (Table 4). Rosette depletion did not affect the response to other noncross-reactive red cells (*Brody*, 1970; *Gorcynzsky* et al., 1971; *Bach* et al., 1970). RFC that could be enriched were mainly B cells since addition of pure T cells was required and sufficient to initiate their response (*Brody*, 1970; *Gorcynzsky* et al., 1971) in an adoptive transfer system. Similar findings were reported by *Osoba* (1970) and *Haskill* and *Marbrook* (1971) who employed tissue culture techniques. RFC of SRC-immunized mice included memory B cells, the response of which also depended on helper T cells (*Wilson*, 1973) at least sometime after immunization. *Sulitzeanu* and *Axelrad* (1973) fractionated rosettes formed by spleen cells from HSA-immunized mice and HSA-coupled SRC. RFC-enriched and RFC-depleted fractions gave enhanced or decreased anti-HSA antibody responses in adoptive recipients if stimulated with HSA-SRC. *Elliott* and *Haskill* (1973) separated larger B rosettes representing AFC from smaller B rosettes including B memory cells. The purpose of all these experiments was mainly to study the function of RFC and not to develop techniques for high purification of specific lymphocytes. It is clear from these studies that RFC include AFCP, although more quantitative data on the frequency of RFC and AFCP in enriched and depleted fractions would be desirable.

More recently, enrichment of turkey γ-globulin (RGG) and SRC-specific lymphocytes from immunized mice was achieved in a rather indirect way by *Walker* et al. (1977). The authors depleted antigen-specific B-lymphocytes of M-Ig by treatment with antigen at 37° C. All M-Ig is capped with antigen under appropriate conditions (*Raff* et al., 1973) and the antigen-receptor complexes are finally shedded from the cell surface (*Ault* and *Unanue*, 1974). Such treatment with antigen followed by removal of M-Ig-positive cells after rosetting

with anti-Ig Fab-coupled red cells resulted in specific enrichment of AFCP in the nonrosetting cells still containing all other M-Ig-negative cells (*Walker* et al., 1977). Recently, hapten-specific RFC present in human peripheral blood were enriched by centrifugation on Ficoll-Isopaque (density 1077) and subsequently infected with EB virus (*Steinitz* et al., 1977). Cell lines were obtained containing 13%–18% hapten-specific RFC. By further purification of renewed rosettes, the frequency of transformed cells forming hapten-specific rosettes as well as hemolytic plaques was increased up to about 80%.

β) Antibody-Forming Cells (AFC)

Specific rosette separation techniques were also used to study the relationship between M-Ig and secreted Ig of AFC. SRC-RFC depleted fractions of immune spleen cells were also depleted in anti-SRC AFC (*Maov* and *Harris*, 1970; *Edwards* et al., 1970). In other studies, depletion of rosettes prepared at 4° C or at 37° C removed only a minor proportion of AFC (*Wilson*, 1971). Multilayer rosettes (clusters) are characteristic for rosette formation by AFC at 37° C (*Duffus* and *Allan*, 1969; *Shearer* and *Cudkowicz*, 1968). Such multilayer rosettes may be separated from single layer rosettes and single cells by velocity sedimentation (*Elliott* and *Haskill*, 1973). Agglutination of SRC around antibody-secreting cells by anti-Ig resulted in the formation of very large clusters that could be isolated by velocity sedimentation (*Lubbe* et al., 1976). Rosette separation techniques can be used to purify AFC; however, the presence of antigen (red cells or their fragments) may limit further studies on the separated cells. Antibody secretion, for instance, appears to be blocked after binding of specific antigen to the plasma cell surface (*Schrader* and *Nossal*, 1974).

b) Separation of T Rosette-Forming Cells (T-RFC)

Antigen-specific rosette formation by T cells is highly controversial (for review, see *Wilson* and *Miller*, 1971; *Miller*, 1972; *Crone* et al., 1972; *Warner*, 1974). The work of *Haskill* et al. (1972) and *Elliott* and *Haskill* (1973) indicated that the main problem may be the instability of T rosettes, which dissociate in a short period of time even at 4° C. The dissociation of SRC from T cells could be prevented by addition of sodium azide after rosette formation and T-RFC could be sparated in the presence of azide from nonrosetting lymphocytes and B-RFC on the basis of their "low binding capacity" by velocity sedimentation. The effect of the metabolic inhibitor was readily reversed by washing with azide-free medium, which allowed functional studies on the T-RFC-enriched fraction in adoptive transfer experiments. T-RFC did not function as helper cells, neither in a primary anti-SRC IgM response (*Elliott* et al., 1973; *Elliott* and *Haskill*, 1975) nor in a secondary IgM and IgG response (*Elliott* and *Haskill*, 1975). Helper function was always associated with rosette-depleted fractions. Other authors, however, claim that SRC-specific T rosettes could be formed and separated in the absence of azide and that they did function as helper cells (*Kontiainen* and *Andersson*, 1975), at least if the lymphocytes were taken from animals immunized 8 days before with SRC (*Wilson*, 1973). The

data of *Bach* and *Dardenne* (1972) were taken to indicate that SRC-RFC include both AFCP and T helper cells.

More recently, *Elliott* et al. (1975) described a medium sized T cell that could form specific rosettes with SRC and mediated delayed hypersensitivity (assayed by transfer of immune cells to SRC-challenged recipients) and in vitro cytotoxicity (assayed by specific killing and growth inhibition of sheep fibroblasts sharing determinants with sheep erythrocytes). *Cone* et al. (1977) also found T-RFC in mice immunized with a low dose of red cells. The T rosettes were unstable like the ones described by *Elliott* and *Haskill* (1973), but the T-RFC adhered to nylon wool in contrast to the majority of the cells responsible for specific DTH reactions. The authors suggested that T-RFC acquired their specificity via antigen-specific products released from T suppressor cells. Experiments performed by *Brondz* et al. (1973a) indicate that cytotoxic T cells specific for allogeneic cells do not form rosettes after overnight incubation at 4° C with red cells carrying or coupled with the relevant alloantigens. All studies on separation of antigen-specific T-RFC are not really satisfying, particularly because of insufficient quantitative data on the purity of T-RFC-enriched fractions and their functions.

F. Electronic Cell Sorting

Electronic cell-sorting instruments have been developed in the past decase originally for separation of cells according to gross physical parameters. The instruments available are based on the deflection of cells according to cell surface charge (*Hannig*, 1964, 1971) or on the electrostatic deflections of charged droplets containing single cells (*Fulwyler*, 1965; *Fulwyler* et al., 1969). Specific cell separation by preparative cell electrophoresis has been suggested (*Rhie* and *Sehon*, 1972) but has never been successful. The further development of the droplet separation system and combination with orther instruments allowing the observation of fluorescence and other optical characteristics of cells in flow systems (*Kamentsky* et al., 1965; *Van Dilla* et al., 1969) have led to the fluorescence-activated cell sorter (FACS), which allows separation of cells according to size and intensity of fluorescence associated with single cells (*Hulett* et al., 1969). The FACS can be used for analytic as well as preparative purposes (*Horan* and *Wheeless*, 1977). Other instruments are currently being developed for separation of cells by means of a magnetic field technique that may also be applicable in future for the separation of antigen-specific lymphocytes (*Roth*, 1977). The following brief description of the FACS and its application for specific lymphocyte separation is based on the literature available and our rather limited experience with the FACS at the Basel Institute for Immunology.

1. Fluorescence Staining of Antigen-Specific Cells

Coons et al. (1941) first described the possibility of fluorescence labeling of specific antibodies without affecting the antigen-binding capacity so that they could be used to detect various antigens in tissues. Immunofluorescence tech-

niques were improved considerably when new fluorescent reagents and appropriate conjugation conditions were developed. The basic procedures were described recently in detail by *Forni* (1978), who also gives references to the relevant literature. Fluorescence staining techniques are considerably less sensitive than radioautographic methods, which is probably the reason why the use of fluorescence-labeled antigens or antibodies for the detection of specific receptor-bearing lymphocytes has been rather limited until the development of the fluorescence-activated cell sorter (FACS). Antigen receptor-bearing lymphocytes can be labeled with fluorescence in several ways:

1. Cells are first exposed to antigen in vitro (*Julius* et al., 1972, 1975, 1976; *Julius* and *Herzenberg*, 1974) or in vivo (*Scott*, 1976a, b) and then, after washing, incubated with fluorescence-labeled specific antibodies.

2. Cells are incubated with fluorescent-labeled protein antigens (*Julius* et al., 1972) or hapten-protein conjugates (*Julius* and *Herzenberg*, 1974). Obviously, cells specific for haptens that are fluorescent themselves are best suited for specific separation in the FACS (*Nossal* et al., 1978). As carriers, autologous proteins that have a low tendency to bind nonspecifically to lymphocytes should be used. With immunoglobulins as carriers and/or labeling reagents (*Julius* et al., 1972; *Julius* and *Herzenberg*, 1974), care has to be taken to avoid nonspecific labeling of Fc receptor-bearing cells.

3. Anti-idiotypic antibodies may be used to label directly or indirectly and to purify idiotype-bearing cells. Idiotype-specific fluorescence labeling of lymphocytes has been demonstrated (*Köhler* et al., 1974; *Binz* and *Wigzell*, 1975b; *Krammer* and *Eichmann*, 1977) and is currently used by *Julius* and collaborators (1977, personal communication) in attempts to purify antigen-specific T-lymphocytes.

4. Intensively fluorescent microspheres coupled with antigens or antibodies may also be used in future to label and separate specific lymphocytes (*Roth*, 1977).

2. Separation of Fluorescence-Labeled Cells

The first fluorescence-activated cell sorter has been developed by *Bonner* et al. (1972) and *Hulett* et al. (1969, 1973). A modified version is now commercially available from Becton-Dickinson Electronic Laboratories (*Loken* and *Herzenberg*, 1975). This instrument operates on a dual channel mode. The fluorescence channel is activated only when a light scatter signal indicates that a cell is present. Scatter and fluorescence signal ranges can be specified independently to define two cell populations A and B that can be separated from each other by a system of charged droplet deflection in opposite directions similar to that described earlier by *Fulwyler* (1965).

Cells that do not meet the A or B criteria (for instance, dead cells that give a characteristic light scattering) or cells too closely spaced in the flow system to be separated properly in one droplet are discarded in the nondeflected droplet fraction. For analytic purposes, 500–1000 cells may be processed per second. For preparative cell separations, the flow rates may be increased up

to 5000 cells per second, i.e., approximately 2×10^7 cells per hour (*Hulett* et al., 1973; *Bonner* et al., 1972).

Separations of specific cells comprising only a small fraction of lymphoid cell suspensions is best accomplished using a double pass procedure (*Julius* et al., 1972). First a preliminary separation at a very high flow rate (e.g., 20000 cells per second or even more) is used without discarding closely spaced cells. The enriched population is then concentrated by centrifugation and separated again at lower flow rates to achieve the desired final purity of specifically labeled cells. Three aspects of fluorescence-activated cell sorting should be discussed briefly here.

1. Little information on the total cell recoveries is available in the current literature on the FACS.

2. The resolution of the droplet separation system is sufficiently high to achieve highly purified cell populations, at least at relatively low flow rates. The separation time required for obtaining 10^5 cells (frequency 10^{-4}) is several hours. The separation time may be limiting if the fluorescence label is lost from the cells with time of incubation. This is not the case with specific fluorescence-labeled antigen-binding B-lymphocytes if they are separated at 4° C and in the presence of NaN_3 (*Julius*, personal communication). Shorter separation times will probably be sufficient in future since more and more techniques are becoming available for functional studies on very small numbers of cells. The separation time required to obtain a sufficient number of purified cells may be reduced when prefractionated cells are used (*Nossal* et al., 1978).

3. The minimum number of fluorescein molecules that the FACS can detect above background is estimated to be between 3000 and 5000 (*Loken* and *Herzenberg*, 1975; *Hulett* et al., 1973). There must be an extensive overlap in fluorescence intensity of specifically and nonspecifically stained cells that limits the purification of specific receptor-bearing cells. This problem is less pronounced but not eliminated if only high affinity receptor-bearing cells are stained with low concentrations of antigen (*Julius* and *Herzenberg*, 1974). For purification of specific antigen-*reactive* cells, the optimal antigen concentrations for staining and the optimal threshold setting of the fluorescence channel have to be elaborated.

3. Applications

a) Separation of Specific B Cells

Antigen-Binding Cells

Julius et al. (1976) enriched populations of antigen-binding cells employing the FACS in order to study the specificity of receptors expressed on single lymphocytes. The separation of fluorescein-DNP mouse γ-globulin (^{F}DNP-MGG) binding cells and rhodamine-KLH (^{R}KLH) binding cells was incomplete, and the distinction of cells binding only one or both antigens was dependent on visual inspection of single cells. The statement that ABC stained with 167 µg/ml $^F DNP_{23}$-MGG included all potential anti-DNP AFCP in normal spleen cell populations (*Julius* et al., 1976) is in contrast to the data described in

the cited publication (*Julius* and *Herzenberg*, 1974), which indicated that *no apparent depletion* of the anti-DNP PFC response was obtained with cell populations depleted of ABC (stained with 167 μg/ml $^{F}DNP_{23}$-MGG).

^{F}DNP-MGG binding cells were considered to be DNP-specific, although 50% inhibition of binding was observed in the presence of free MGG. Binding to fluorescein-specific cells was neglected. The fact that the cells binding ^{F}DNP-MGG (about 1% of all normal spleen cells) and the cells binding ^{R}KLH (about 0.5% of all normal spleen cells) were nonoverlapping populations does not provide evidence for monospecificity of antigen-binding cells.

Other studies indicated that single lymphocytes can express antigen receptors with at least two different specificities (*Miller* et al., 1971) while studies by *Raff* et al. (1973) suggested that all Ig expressed at the surface of a single lymphocyte have the same specificity. The limitations of both experimental approaches cited were discussed by *Nossal* (1974). It should be stated clearly that the expression of receptors for more than one antigen by single lymphocytes has not been excluded yet.

Antibody-Forming Cell Precursors From Normal and Immunized Mice

Julius et al. (1972) have shown that FACS-enriched antigen-binding cells present in the spleen from immunized mice include precursors of antibody-forming cells. In a first series of experiments, fluorescein-conjugated KLH (^{F}KLH) or human serum albumin (^{F}HSA) binding cells were purified and injected into irradiated syngeneic recipients. As few as $3–5 \times 10^4$ purified cells gave significant anti-KLH or anti-HSA antibody responses, provided T helper cells were also injected (*Julius* et al., 1972).

In a second series of experiments, it was demonstrated that purified fluorescein-DNP-MGG (^{F}DNP-MGG) binding spleen cells from unprimed mice included anti-DNP AFCP (*Julius* and *Herzenberg*, 1974; *Julius* et al., 1975). Selective staining of high, medium, and low avidity DNP-binding cells was performed in attempts to separate the precursors of cells secreting high, medium, and low affinity anti-DNP antibodies from each other and from the great majority of other lymphocytes.

More recently *Nossal* et al. (1978) used fluorescein (F)-conjugated gelatin to label fluorescein specific B-cells. ^{F}Gelatin binding cells were first separated by the hapten-gelatin separation procedure (see above) and subsequently further purified with the aid of the FACS. One out of 8 purified cells was stimulated in limiting dilution cultures by fluorescein conjugated onto polymerized flagellin, a T-cell independent carrier. The authors state in the discussion that "the numbers of observed ABC and their apparent monospecificity agreed well with recent findings by *Julius* et al. (1976)". In the study of *Julius* and *Herzenberg* (1974) and *Julius* et al. (1976) the cells labeled with fluorescein after incubation with $F_{3.5}$-DNP_{23}-MGG (167 μg/ml) were believed to be *DNP* specific. However, one would expect that as many F-DNP-MGG binding cells were fluorescein specific as were labeled by F_4-gelatin (100 μg/ml) in the study of *Nossal* et al. (1978). Obviously *Julius* and *Herzenberg* (1974) and *Julius* et al. (1976) could not determine wether AFCP are monospecific or not.

b) Separation of Specific T Cells

All fluorescence-labeled ABC tested were θ-negative, surface Ig-positive B-lymphocytes (*Julius*, personal communication). Moreover, the response of purified KLH-binding cells from immunized mice was dependent on the addition of helper cells that were found in the cell populations depleted in ^{F}KLH-ABC (*Julius* et al., 1972). These data indicate that T helper cells bind significantly less antigen than AFCP or that the antigen binding to T cells is not stable under the separation conditions used.

More recently, *Julius* and collaborators succeeded in separating, with the aid of the FACS and anti-idiotypic antisera phosphorylcholine (PC), specific T helper cells bearing the TEPC 15 idiotype from DNP-specific helper cells and the majority of other T-lymphocytes. Enrichment but not depletion of PC-specific T helper cells was also achieved by separation of fluorescence-labeled PC-BSDA-binding cells (*Julius*, 1978, personal communication).

G. Elimination or Inactivation of Specific Lymphocytes by Radioactively Labeled Antigen or Antibody

1. Techniques

Antigens labeled with a soft β-ray-emitting isotope may be used for selective killing or inactivation of antigen-binding cells. If specific receptor-bearing cells were to be selectively irradiated without damaging neighboring cells, the radiation would need to be largely absorbed within about 5 μm but also to penetrate for at least 1 μm so as to traverse the thin layer of the cytoplasma of a lymphocyte to reach the nucleus (*Humphrey* et al., 1971). For this purpose, as for high resolution autoradiography, tritium (^{3}H) or ^{125}I are well-suited since these isotopes emit mainly short range β-rays. For hot antigen suicide experiments, ^{125}I was used by all investigators since the carrier-free isotope can be readily introduced into proteins using chloramine T as the oxidizing agent (*Hunter* and *Greenwood*, 1962) and since ^{125}I has a much shorter half-life than ^{3}H.

Humphrey and *Keller* (1970) found that mice normally responding to TIGAL did not produce anti-TIGAL antibodies after injection with ^{125}I-labeled TIGAL of high specific activity, even not after challenge with unlabeled TIGAL. It was not clear whether the nonimmunogenicity of ^{125}I-labeled TIGAL was due to self-destruction of the antigen. *Ada* and *Byrt* (1969) were the first to clearly demonstrate hot antigen suicide of antigen-reactive B cells. Lymphoid cells were treated with ^{125}I-labeled T-independent antigens, washed, incubated overnight at cold temperatures, and then transferred to irradiated syngeneic recipients that were immunized with unlabeled antigen (for details, see Table 5). Subsequently, similar procedures were used in attempts to eliminate or inactivate other types of antigen-specific lymphocytes.

Table 5. Hot antigen "suicide" of specific T and B lymphocytes

Responder cells	Cells/ml	Incubation with hot Antigen		Temperature	Time	Inactivation of	References
		^{125}I-labeled antigen (conc.)	spec. act. μCi/μg AG				
Mouse spleen	1.3×10^8/6 ml	POL (5–50 μg/6 ml)	25	0° C (+16–20 h at 0–4° C)	30 min	AFCP	*Ada* and *Byrt* (1969)
Mouse Spleen	$1–2 \times 10^7$/ml	KLH, HGG (1–2 μg/ml)	240–1000	2° C	120 min	AFCP (n+i) T helper?	*Unanue* (1971 a)
Mouse bone marrow	$1.5–3.7 \times 10^8$/8 ml	KLH (50–110 μg/8 ml)	194–232	4° C	120 min	AFCP T helper?	*Unanue* (1971 b)
Mouse spleen cells	?	TIGAL, KLH, MSH (2, 4, 5 μg/ml)	>200	0° C (+20 h at 4° C)	60 min	7S, 19S AFCP T helper (yes/no)	*Humphrey* et al. (1971); *Roelants* and *Askonas* (1971)
Mouse (activ.) thymus cells	$3–9 \times 10^7$/ml	POL (1.1–15.7 μg/ml)	50–84	0° C (+14–19 h at 4° C)	30–40 min	DTH T cells (see text)	*Cooper* and *Ada* (1972)
Mouse spleen	5×10^7/ml	DNP proteins (1 μg/ml)	232–250	0° C	120 min	7S, 19S AFCP T helper (yes/no)	*Golan* and *Borel* (1972)
Mouse thymus, bone marrow (TXBM) spleen	FGG 70 ng/10^8 cells		150–170	0° C	30 min	7S AFCP in TXBM: yes; 7S bone marrow: no; T helper in thymus: yes	*Basten* et al. (1971)
Splenic B and T cells (mice)	FGG 10 μg/5×10^6 cells T cells incubated with hot antigen treated B cells (60 min, 37° C+20 h at 4° C)		150	4° C	30 min	7S AFCP: yes; T helper cells: no T helper cells: yes	*Basten* et al. (1975)

2. Applications

a) Inactivation of Specific B Cells

Ada and *Byrt* (1969) incubated normal spleen cells with ^{125}I-labeled polymerized flagellins (POL, SW1 338, or SL871) for 30 min at 0° C in the presence of sodium azide (0.015 *M*) to prevent the uptake of large amounts of antigen by phagocytic cells. The cells were then centrifuged through fetal calf serum underlayers and incubated for 16–20 h at 0°–4° C before transfer into syngeneic-irradiated recipients. The recipients were immunized with both POL-SW1 338 and POL-SL871, and antibody titers were determined 8 days later by the flagella immobilization test. The response to the antigens used during the preincubation was specifically abolished. Normal responses to both antigens were obtained after in vitro pretreatment with cold antigens or when cells treated with hot antigens were transferred into normal nonirradiated recipients, i.e., cells treated with hot antigen did not suppress other cells. These first hot antigen suicide experiments are still the best demonstration that specific AFCP can be inactivated selectively by hot antigen, since T helper cells are not required for anti-POL antibody responses. *Humphrey* et al. (1971) used the same procedure to eliminate anti-TIGAL, hemocyanin (KLH, MSH), and DNP antibody responses. After suicide of DNP-specific clones, propagated in irradiated recipients, several new anti-DNP antibody-producing clones appeared promptly. This effect appeared not to be due to escape from suicide of low affinity receptor-bearing cells (*McMichael* and *Willcox*, 1975; *Willcox* and *McMichael*, 1975).

In other studies, incubation of lymphocytes with hot antigen for 30–120 min at cold temperatures was found to be sufficient for elimination of specific antibody responses. *Unanue* (1971 a) abolished the adoptive antibody response of spleen cells from normal and immunized mice to KLH and HGG. Higher specific activities of the antigens were required for elimination of memory cells than for elimination of virgin precursor cells. The suicide was antigen specific, but it was not clear whether specific B or T cells or both were eliminated. A similar suicide effect was observed with normal bone marrow cells which gave—if not pretreated with hot antigen—a surprisingly high adoptive antibody response to the T cell-dependent antigen KLH (*Unanue*, 1971 b). In contrast, no suicide effect was observed after transfer of thymus cells and bone marrow cells treated with hot FGG, even if ten times as much ^{125}I-FGG was used as was sufficient for inactivation of FGG-specific AFCP in spleens of adult thymectomized and bone marrow protected TXBM mice (*Basten* et al., 1971). *Golan* and *Borel* (1972) abolished adoptive anti-DNP PFC responses of normal spleen cells by treatment for 2 h at 0° C with various ^{125}I-labeled DNP-protein conjugates.

b) Inactivation of Specific T Helper Cells and T Suppressor Cells

Basten et al. (1971) transferred B cells (bone marrow cells from normal mice or spleen cells from adult thymectomized, bone marrow reconstituted TXBM mice) and/or T cells (thymus cells from normal mice) in syngeneic-irradiated

recipients. The adoptive anti-FGG 7S antibody response was clearly dependent on both B cells and T cells. If the thymus cells were treated with 70 ng ^{125}I-FGG per 10^8 cells for 30 min at 0° C before transfer with B cells, no anti-FGG antibody response but normal anti-HRC responses were observed. This apparent T helper cell suicide was not due to in vivo shedding of hot antigen and subsequent B cell inactivation since the suicide was abolished by addition of untreated T cells. Pretreatment of thymus cells for 3 h at 0° C with Fab_2 from rabbit antimouse κ-chains but not from normal rabbit antibodies abolished the suicide (*Basten* et al., 1971).

More recently, *Basten* et al. (1975) found that FGG-specific T helper cells present in anti-Ig column passed spleen cells could be inactivated only by incubation for 1 h at 37° C with ^{125}I-FGG-treated B cells (anti-θ-treated spleen from FGG-primed mice) and subsequent overnight incubation at cold temperatures. The T helper cell suicide but not the B cell suicide was abrogated by pretreatment with the Fab fragments of appropriate anti-H-2 antibodies. This was interpreted to indicate that H-2 (Ir-gene) associated determinants were close to the antigen-binding sites of T cells but not of B cells (*Basten* et al., 1975). Current experiments by *Loblay* and *Basten* (*Basten*, 1978, personal communication) indicated that purified T helper cells obtained 4 weeks after immunization with HGG in adjuvant were not susceptible to suicide with either ^{125}I-labeled HGG or anti-HGG. However, if the cells were pretreated with cold HGG followed by ^{125}I-anti-HGG, suicide took place. The effect was antigen specific. These results were interpreted to indicate that T helper cells bound antigen but at a density insufficient to allow suicide unless cross-linked with antibody. Splenic T suppressor cells obtained 10 days after immunization with deaggregated HGG were inactivated after incubation with ^{125}I-IgG anti-HGG but not after incubation with ^{125}I-HGG, indicating that HGG was bound to the cell surface of suppressor cells. The hot antibody suicide of T suppressor cells was prevented by pretreatment with cold anti-HGG or with anti-I a antibodies.

c) Inactivation of Specific DTH T Cells

Cooper and *Ada* (1972) transferred normal thymus cells in irradiated syngeneic recipients that were challenged intravenously with polymerized flagellins (POL-870 or POL-1338). After 7 days, spleen and lymph nodes containing POL-activated T cells were injected intravenously into normal mice that received POL into the foodpads 1–2 h later. The activity of the transferred POL-activated T cells was assessed by measurement of the footpad thickness as compared with the footpad thickness of normal mice receiving only POL. Treatment of the normal thymus cells and of the POL-activated T cells with ^{125}I-POL or with anti-θ serum and complement but not with cold POL significantly reduced the DTH. The hot antigen treatment was performed at cold temperatures for 30–40 min. After removal of excess hot antigen, the cells were incubated for further 14–19 h at 4° C. The two serologically distinguishable POL antigens (870 and 1338) were apparently not distinguished by DTH T cells. Treatment with ^{125}I-hemocyanin did not abolish the anti-POL DTH response, but it was not demonstrated that it abolished an antihemocyanin DTH response. Pretreat-

ment of the cells with antilight-chain antibodies prevented that hot POL-induced suicide.

III. Positive and Negative in Vivo Selection of Specific Lymphocytes

A. Introduction

A large number of experiments suggests that antigen-reactive T and B cells migrate to tissues where the antigen is localized, such as lymph nodes or spleen, depending on the route of immunization. After activation by antigen, many specific cells reenter the recirculating lymphocyte pool. The rather complex field of the migration behavior of lymphocytes has been reviewed in great detail by *Ford* (1975) and *Sprent* (1977). Here we describe briefly only the basic experimental protocols which offer the opportunity of obtaining cell populations enriched or depleted in specific antigen-reactive lymphocytes particularly of the T cell class.

B. Antigen-Specific Lymphocyte Recruitment (ASLR)

1. ASLR in Animals Injected With Antigen

a) Specific Depletion of Antigen-Reactive Lymphocytes

Sprent et al. (1971) first demonstrated that 1–2 days after intravenous application of antigen the recirculating lymphocytes were unresponsive to the injected antigen but not to other antigens. The effect was demonstrated for both B cells and T helper cells in mice injected with a large number of heterologous erythrocytes (*Sprent* et al., 1971; *Sprent* and *Miller*, 1974; *Sprent* and *Lefkovits*, 1976; *Sprent*, 1977) and also in rats injected with hapten carrier conjugates (*Rowley* et al., 1972). *Sprent* and colleagues demonstrated convincingly that in mice the unresponsiveness of thoracic duct lymphocytes (TDL) as well as of mesenteric lymph node cells early after intravenous application of antigen was indeed due to ASLR to the spleen where the majority of the antigen was localized (*Sprent* and *Miller*, 1974; *Sprent* and *Lefkovits*, 1976) and not to the rapid generation of specific suppressor cells as described by *McCullagh* (1975) who performed similar experiments with rats. In the original studies of *Sprent* et al. (1971) and *Rowley* et al. (1972), ASLR was also shown for HvG-reactive lymphocytes in parental strain mice injected with their F1 hybrid spleen cells. In these experiments, some injected cells recirculated at least initially and thereby "contaminated" the host TDL. More recently, *Sprent* and *Miller* (1976a) injected spleen cells which were cultured in vitro for several hours after irradiation (1000 rad). This treatment allowed the great majority of the injected cells to localize to the spleen and prevented them from recirculating (*Andersson* et al., 1974). Injection of large numbers of irradiated F1 hybrid cells

($2–6 \times 10^6$) into parental strain mice specifically abolished the capacity of TDL, collected 1 day later to respond in the GvH reaction, skin graft rejection, and CML to the antigens present on the injected cells. The capacity of the TDL to proliferate in vivo or in vitro (*Sprent*, 1973) upon stimulation with the injected F1 hybrid cells was reduced only twofold to threefold even after injection of as many as 6×10^8 irradiated semiallogeneic cells (*Sprent* and *Miller*, 1976).

ASLR was also observed in studies on isolated sheep lymph nodes (*Hay* et al., 1974). Lymphocytes were collected from the efferent vessel of a popliteal sheep lymph node after local injection of soluble antigens or allogeneic lymphocytes. Three days after the injection, the lymph node effluent cells were specifically unresponsive to the injected cells or antigens. In some experiments, injection of 5×10^8 allogeneic lymphocytes into the afferent lymph vessel was sufficient to cause a complete depletion of MLC-reactive lymphocytes in the efferent lymph. In all situations described above, injection of a high dose of antigen was required to obtain complete disappearance of antigen-reactive cells from the recirculation. With a low dose of antigen, probably only high affinity receptor bearing cells were recruited (*Sprent*, 1977).

b) Enrichment of Antigen-Reactive Lymphocytes

ASLR should not only lead to depletion of antigen-reactive lymphocytes in the recirculating pool but also to an accumulation of antigen-reactive cells at the site of antigen localization. This has been demonstrated in many situations (for review, see *Sprent*, 1977). Depending on the route of antigen administration, lymph nodes or the spleen can serve as a source of cell populations relatively enriched in lymphocytes recently activated by antigen. It should be noted, however, that some properties of lymphocytes may be drastically altered in the antigen-activated state. *Sprent* and *Miller* (1973), for instance, found that the spleen cells from mice injected intravenously with antigen 1–2 days before were unable to respond to the injected antigen within 1 week after adoptive transfer but responsiveness returned if the challenge was delayed for 5 days (*Sprent* and *Miller*, 1973). Rapid and above normal responses of these cells were found in vitro (*Sprent* and *Lefkovits*, 1976) indicating that the unresponsiveness observed in vivo was probably due to a temporary alteration in the migration properties of the recently activated cells.

Allografts were shown to retain specifically alloreactive host lymphocytes (*Lance* and *Cooper*, 1972). Recently, an experimental model was described that made it possible to recover allograft infiltrating host cells (*Roberts* and *Häyry*, 1976). Viscous cellulose sponges were implanted in the peritoneal cavity of DBA/2 or CBA mice (primary hosts). The sponge that was infiltrated within 5–7 days mainly with fibroblasts was then transplanted to the neck or the peritoneal cavity of CBA mice (secondary host). The sponge transplants were removed 8–10 days later and the infiltrated cells were released by compression. The "allogeneic" sponge graft but not the "syngeneic" sponge graft had induced a virgorous inflammatory reaction and was heavily infiltrated by host lymphocytes, macrophages, and granulocytes. These cells were cytotoxic for target cells derived from the primary allogeneic host (*Roberts* and *Häyry*, 1976). Similar

experiments were performed in rats by *Binz* et al. (1976). These authors found that 1 week after transplantation of a fibroblast infiltrated sponge into an allogeneic host about 40% of the cells recovered from the sponge bound anti-idiotypic antisera directed against host recognition structures for the grafted cells. A large proportion of these idiotype-positive cells ingested latex particles and were considered to be macrophages coated with idiotypic molecules (*Binz* et al., 1976; *Binz* and *Wigzell*, 1977b).

2. Antigen-Specific Recruitment of Lymphocytes Injected in Antigen-Bearing Recipients

a) Specific Depletion of Antigen-Reactive Lymphocytes

ASLR was also observed when parental strain lymphocytes from the recirculating pool (TDL or lymph node cells) were injected into their irradiated semiallogeneic F1 hybrids. In earlier studies by *Sprent* and *Miller* (1971, 1972a, b, c), recruitment of host-reactive donor cells must have occurred within the first 3 days after transfer, but at this time the number of recirculating parental cells was too low for functional test because only 2×10^6 parental TDL were injected. *Ford* and *Atkins* (1971) circumvented this difficulty by transferring large numbers of rat TDL (2×10^9) into F1 hybrid recipients that were irradiated 3 days before in order to reduce the number of recirculating cells of recipient origin. Approximately 75% of the TDL collected 12–36 h after the injection were donor-type cells. The GvH reactivity of these cells as tested by the popliteal lymph node assay was profoundly depressed in F1 hybrid rats syngeneic to the filter rat but normal in hybrids expressing third party histocompatibility antigens. These findings were confirmed by others (Table 6) and currently represent the most efficient technique for the removal of specific alloreactive T cells from a population of lymphocytes. The experimental protocol can also be adapted to deplete T cells reactive to conventional non-MHC antigens. For example, *Sprent* has found that T cells filtered from blood to lymph for 1 day through irradiated syngeneic mice injected with SRC specifically loose their helper function for humoral immune responses to this antigen (*Sprent*, 1978a, b).

b) Enrichment of Antigen-Reactive Lymphocytes

In the filtration experiments described above, the antigen-reactive cells were recruited to the spleen, providing a rich source of recently activated T cells. Only the donor cells are functional in the host spleen, while the host-derived cells are inactivated by irradiation and can be removed in the case of the parent F1 experiments by treatment of cell suspensions with antisera and complement. It is clear, however, that cells not specific for host determinants are also found in the spleen in addition to ASLR (*Sprent*, 1977).

Ford and *Atkins* (1971) found in their original experiments that the GvH reactivity of parental TDL recruited to the spleen of "filter" rats (removed 12 h after injection) did not differ significantly from that of normal parental

Table 6. Specific lymphocyte recruitment of parental cells in F1 Hybrids

Parental donor cells	F1 hybrid recipient (irradiated with)	Early TDL collection (depleted in)	Studies on	References
Rat TDL (up to 2×10^9)	400–450 rad	GvHR	Frequency of alloreact. T cells	*Ford* and *Atkins* (1971, 1973); *Atkins* and *Ford* (1975); *Ford* et al. (1975)
Rat TDL (10^9)	900–1000 rad	GvHR adopt. allograft rej.	Precommitment of alloreact. T cells	*Dorsch* and *Roser* (1974)
Rat TDL	450 rad	GvHR MLR (in vitro)	T-B cooperation	*Heber-Katz* and *Wilson* (1975)
Mouse lymph node (10^8)	1000 rad	MLR (in vivo)	Affinity of MLR T cells?	*Sprent* and *Miller* (1976a)
Mouse lymph node (10^8)	1000 rad	MLR (in vitro)	T-B cooperation	*Sprent* and *von Boehmer* (1976)
Rat TDL (1.5×10^9)	400 rad	GvHR, MLR, CML	Alloreact. T cells	*Wilson* et al. (1976a, b)
TDL (rats) lymph node (mice)	450 rad 900 rad	MLR (in vitro)	CTL to haptenated cells	*Wilson* et al. (1977)

TDL injected either in "filter" rats or F1 hybrids expressing "third party" histocompatibility antigens. The absence of hyperreactivity may in this case also be due to the alteration of migration behavior after activation or alternatively to exhaustive proliferation (see below).

3. Applications

a) Precommitment and Frequency of Alloreactive T Cells

The phenomenon of ASLR supports the concept that T cells involved in GvHR, MLR, CML, and skin graft rejection are precommitted to recognize one particular allogeneic determinant or set of determinants controlled by the MHC. There have been serious doubts on the specificity restriction of alloreactive T cells because of the high frequency of lymphocytes reactive to one particular type of allogeneic cells (*Szenberg* and *Warner*, 1961; *Simons* and *Fowler*, 1966; *Simonsen*, 1967; *Nisbet* et al., 1969). *Ford* and collaborators (*Ford* and *Atkins*, 1973; *Atkins* and *Ford*, 1975; *Ford* et al., 1975) investigated the quantitative aspects of recruitment of alloreactive cells. From the extent of the recruitment to the spleen and the proportion of responding cells, the authors estimated that at

least 4.5%–6.0% but not more than 12% of AO rat lymphocytes recognize the strong transplantation antigens determined by the Ag-B locus in HO and DA rats.

b) Use of TDL Specifically Depleted in Alloreactivity

Normally non-MHC antigens are recognized by T cells in association with self-MHC products. For cytotoxic T cell responses to non-MHC antigens, certain self-MHC determinants must be shared by stimulator and target cells (for review, see *Zinkernagel* and *Doherty*, 1977; *Shearer* et al., 1977; *Bevan*, 1976; *von Boehmer* et al., 1976). Similarly, T cells and B cells must share certain MHC products for effective collaboration in humoral immune responses (*Katz* et al., 1973, 1974). For the analysis of the "MHC restriction" phenomenon in immune responses to non-MHC antigens, it is of considerable interest whether T cells can recognize conventional antigens also in association with allogeneic MHC products. This question is difficult to answer because of the strong reaction to allogeneic MHC products masking and/or strongly influencing the responses to non-MHC antigens. Several authors have taken advantage of the very efficient technique of filtering parental strain lymphocytes through irradiated F1 hybrids for removal of a specific population of alloreactive lymphocytes. The parent through F1 filtration may be preferred for most purposes because the depletion of MLR reactivity in parental animals injected with F1 cells is incomplete (*Sprent* and *Miller*, 1976a). The following results were obtained:

1. T-B cell collaboration in an in vitro anti-SRC IgM response was as efficient in allogeneic T-B cell combinations (using negatively selected rat TDL as a source of T helper cells) as in syngeneic T-B cell combinations (*Heber-Katz* and *Wilson*, 1975). These results were not confirmed, however, by *Sprent* (1978a) who used negatively selected mouse TDL as a source of allogeneic helper cells. In chimeric mice, however, T cells acquire the potential to interact with allogeneic B cells (*von Boehmer* et al., 1975b; *Waldmann* et al., 1975b).

2. Rat and mouse TDL specifically depleted in alloreactivity and stimulated with TNP-coupled allogeneic cells lysed TNP-coupled targets sharing MHC determinants with the stimulator cells but lysed not at all or less efficiently syngeneic TNP-coupled targets (*Wilson* et al., 1977). However, the TNP killing system is likely to be an exceptional case because the MHC restriction is not always as strict as in cytotoxic T cell responses to other non-MHC antigens (*Shearer* et al., 1975), and strong cross-reactivities of T cells cytotoxic for allogeneic and TNP-coupled syngeneic cells have been observed (*Burakoff* et al., 1976; *Billings* et al., 1977; *Lemonnier* et al., 1977).

Experiments using the same techniques for depletion of alloreactivity indicated that cytotoxic T cells directed against other haptens or H-Y antigen do not recognize and respond to these antigens if presented by allogeneic stimulator cells (*von Boehmer* et al., 1978a). Experiments with chimeric mice (*von Boehmer* et al., 1975a), however, have shown that the ability to recognize non-MHC antigens in association with allogeneic MHC products can be acquired (*von Boehmer* and *Haas*, 1976; *Zinkernagel*, 1976a, b; *Pfizenmaier* et al., 1976; *Zinkernagel* et al., 1978a, b).

C. Enrichment of Antigen-Stimulated Lymphocytes in the Recirculation in the Postrecruitment Phase

1. Experimental Protocols

In both models considered above, the initial sequestration of antigen-reactive cells in the lymphoid tissues is followed by a stage of positive selection where the progeny of the responding cells enters the recirculation in large numbers (for details, see *Sprent* 1977). This is particularly conspicuous in the case of transferring parental strain T cells to irradiated MHC incompatible recipients: 4–5 days after the transfer, 90% or more of the cells in thoracic duct are blast cells of donor origin. They were called T-TDL (*Sprent* and *Miller*, 1972a) since the great majority (95% or more) were T cells. Further experiments of *Sprent* and his colleagues (for review, see *Sprent*, 1977) indicated that these blast cells represent an expanded population of activated T cells generated from precursor T cells of donor origin with specificity for host antigens. Thus, T-TDL represent a cell population highly enriched in activated T cells, the specificity of which depends on the antigenic difference between the donor and the recipient. Similarly, 5 days after injection of T cells and heterologous red cells to irradiated syngeneic mice, the thoracic duct lymph is greatly enriched in SRC-specific helper T cells (*Sprent*, 1978a, b).

2. Applications

a) T-TDL as a Source of Highly Enriched Specific T Cells

Sprent and *Miller* (1972a) considered T-TDL already in their original description as a potential source of highly purified T cells activated to antigen. In most of their studies on mouse T-TDL, allogeneic systems were used comprising differences in the whole H-2 complex (for instance, CBA thymus injected in CBA × C57B1/6 or CBA × DBA). The main results are summarized briefly to indicate the potential applications of T-TDL.

1. T-TDL incorporated a substantial amount of ^{3}H-thymidine in vivo (*Sprent* and *Miller*, 1972b) or in vitro (*Cheers* et al., 1974) if reexposed to the original stimulating determinants. As much as 25% of T-TDL synthesized DNA (*Cheers* et al., 1974), but the proliferative potential of T-TDL appeared to be exhausted: most T-TDL did not divide after reactivation in vitro (*Cheers* et al., 1974; *Wilson* et al., 1976a) and failed to produce splenomegaly in recipient type F1 hybrids (*Sprent* and *Miller*, 1972c; *Wilson* et al., 1976a).

2. T-TDL lysed appropriate target cells and suppressed very efficiently the growth of tumor cells bearing the sensitizing alloantigens (*Sprent* and *Miller*, 1972c).

3. T-TDL transferred to B mice recirculated only for a few days and did not home to lymph nodes like normal TDL (*Sprent* and *Miller*, 1976b, c). The majority of T-TDL localized in the gut and probably died (*Sprent*, 1976). A small proportion continued to recirculate for at least 3 months. These long-lived recirculating T-TDL appeared to be positively selected for specific respon-

siveness in MLR, GvHR, and skin graft rejections at least in qualitative terms (i.e., they responded only to the original sensitizing determinants) but not in qualitative terms (i.e., the magnitude of their response was not significantly different from the response of nonsensitized TDL parked in B mice (*Sprent* and *Miller*, 1976c). In contrast, parking in B rats of in vitro activated rat TDL gave highly enriched specific responses upon reexposure to the original sensitizing alloantigens (*Howard* and *Wilson*, 1974; *Wilson* et al., 1976a, b).

4. T-TDL express Ia antigens but no Fc receptors (*Krammer* et al., 1975). A large proportion of T-TDL derived from thymus cells injected into irradiated F1 hybrids were surface IgM and IgG positive. However, almost no surface Ig was detected on T-TDL if the thymus cell population was depleted of B cells (0.9%–0.08%) before injection (*Hudson* et al., 1974; *Sprent* and *Hudson*, 1973).

Surface Ig-negative T-TDL were still cytotoxic for targets bearing the alloantigens of the recipient hybrid suggesting that surface Ig may not be required for antigen recognition (*Hudson* et al., 1974). It was suggested that T-TDL bound specific alloantigen (via an unknown receptor) to which specific anti-alloantibodies of donor B cell origin were subsequently bound. This model was confirmed by *Nägy* et al. (1976a, b) and *Elliott* et al. (1977) who studied in vitro activated T cells. The difficulties involved in testing for enrichment of specific T cells of a particular class and specificity are described in detail in Section IV.

IV. Positive and Negative Selection of Specific Lymphocytes in Tissue Culture

A. Introduction

Positive selection of antigen-reactive lymphocytes occurs in tissue culture on the basis of multiplication and selective survival of stimulated cells. This effect has been used, in some cases in combination with specific and nonspecific cell separation techniques, to purify T cells reactive to a particular alloantigen or set of alloantigens (Sect. IV.B) or to purify antigen-specific delayed-type hypersensitivity (DTH) T cells (Sect. IV.C). Both types of cells are well-suited for positive selection since they proliferate upon stimulation with antigen and differentiate into so-called secondary lymphocytes which are sensitive to restimulation by antigen.

T helper cells for humoral immune responses were separated using antigen-pulsed macrophages (Sect. IV.D), but the stimulation, growth, and differentiation of T helper cells in tissue culture is so far completely obscure. Continuous growth of B cells in vitro has been achieved (Sect. IV.E), but positive selection of antigen-specific B cells in tissue culture has not yet been attempted. Negative selection or elimination of specific lymphocytes in which DNA synthesis can be induced in vitro upon stimulation with antigen has been obtained in short-term cultures with a good survival rate of nonstimulated cells (Sect. IV.F).

B. Positive Selection of Alloreactive T Cells

Proliferative (MLR) and cytotoxic (CML) immune responses to allogeneic cells are the strongest and most reproducible T cell responses obtained in tissue culture. Such responses are usually studied in one-way mixed leukocyte cultures (MLC) containing responder cells and irradiated allogeneic stimulator cells. The proliferative response is most commonly measured by either ^{3}H-TdR incorporation or the cytotoxic T cell (CTL) response in short-term cytotoxicity assays using ^{51}Cr-labeled target cells. A detailed description of these techniques is beyond the scope of this article (for review, see *Cerottini* and *Brunner*, 1974; *Nabholz* et al., 1974; *Meo*, 1978).

Wilson and collaborators (*Wilson* et al., 1972; *Howard* and *Wilson*, 1974) demonstrated that rat TDL parked in T cell-deficient recipients after stimulation in MLC were relatively enriched for T-lymphocytes expressing specificity for the stimulator cells. More recently, several attempts were made to improve the degree of enrichment of alloreactive T cells by using purified T cells as responder cells, by isolation of transformed lymphoblasts briefly before or at the peak of the proliferative response, and/or by prolongation and repeated restimulation of cells in MLCs. The following description of such procedures cannot be separated from a critical evaluation of the experiments providing evidence for positive selection of specific alloreactive T cells.

1. Isolation of Alloantigen-Activated T Cell Blasts

a) Techniques

The preparation of purified alloantigen-activated T cell blasts may be divided into three steps: 1) purification of responder T cells, 2) stimulation in MLC, and 3) isolation of blasts.

1. T cells from various sources (in most cases from mouse spleen) were purified by filtration through nylon wool clumns (*Häyry*, 1976) or through Ig-anti-Ig glass bead columns (*Peck* et al., 1977a, b). Despite the fact that the great majority of lymphoblasts generated in MLC are T cells, highly purified T cells should be used as responders if the isolation of alloantigen-activated T cell blasts is intended and particularly if the isolated blasts should be free of B cell-derived Ig (*Nägy* et al., 1976a).

2. Stimulation of responder cells with irradiated or mitomycin-treated allogeneic cells was usually performed in ordinary MLC. In most cases, responder and stimulator cells from mice differing in the whole MHC region were used (*McDonald* et al., 1974a, b; *Wagner* and *Röllinghoff*, 1976; *Häyry*, 1976). Responses to non-MHC controlled cell surface antigens were also studied (*Peck* et al., 1977a, b; *von Boehmer*, 1977). If purification of specific T cells is intended, the responder-stimulator combinations should be selected carefully to meet only the minimal requirements for stimulation of T cells of one particular specificity. Cells from congeneic recombinant mouse strains should be used as stimulators and responders.

3. For the blast isolation, most authors used the 1 *g*-velocity sedimentation technique as described by *Miller* and *Philipps* (1969) or slightly modified. Briefly, cells from MLC were harvested close to or at the peak of the proliferative response, washed once, and resuspended in DMEM supplemented with 3% FCS at a concentration of $0.5–2 \times 10^6$ cells/ml (*McDonald* et al., 1974b). Thirty milliliters of the cell suspension were applied to a buffered step gradient (7%–30% FCS in DMEM) and allowed to sediment for 4 h at 4° C. Fifteen-milliliter fractions were then collected and concentrated by centrifugation. This procedure with only minor modifications was also used by *Andersson* and *Häyry* (1975), *Wagner* and *Röllinghoff* (1976), *Peck* et al. (1977a, b), and *Andersson, L.C.* et al. (1977).

b) Characterization of Blasts Generated in MLC

α) Heterogeneity

If purified T cells were used as responders, close to 100% of the blasts at the peak of the primary response were found to be T cells (*Häyry*, 1976; *Peck* et al., 1977a). This T cell blast population can be expected to be a rather heterogenous mixture of cells with various specificities and functions. Even after stimulation of purified T cells with cells differing from the responders in a single serologically defined MHC determinant (Ia-7), at least three types of lymphoblasts could be distinguished with anti-Ly antisera (*Peck* et al., 1977a). The "contamination" by large phagocytic nonlymphoid cells and small lymphocytes was marginal (*Peck* et al., 1977a).

β) Cytotoxic Activity

Four to six days after initiation of a primary MLC, about 50% of all cultured cells but more than 90% of CTL were medium size to large cells sedimenting at 4 mm/h (*McDonald* et al., 1974b; *Wagner* and *Röllinghoff*, 1976). Maximum enrichment of cytotoxic activity was found in fractions sedimenting at 6–7 mm/h containing on a per cell basis about 2.5 times more CTL than unfractionated MLC cells (*McDonald* et al., 1974b). These data indicate that the majority of blasts generated by stimulation with cells differing from the responders in the entire MHC are not cytotoxic T cells.

γ) Restimulation of Isolated Blasts

Originally, *McDonald* et al. (1974b) found that day 4 primary MLC cells were insensitive to restimulation. The failure of day 4 primary MLC cells to respond to the original stimulator cells may be related to the presence of cells in primary MLC which either eliminate antigen (*Fitch* et al., 1976) or which suppress responder cells (*Rich* and *Rich*, 1974; *Hirano* and *Nordin*, 1976). Simple dilution of day 4 MLC cells (still containing many cytolytic T cells) permitted restimulation when an excess of stimulator cells was used (*Fitch* et al., 1975).

Restimulation of day 3 secondary MLC cells resulted only in a transient increase in cytotoxic activity for 2 days and a subsequent decline at all responding cell doses tested (*McDonald*, 1978). In contrast restimulation of day 6 secondary MLC cells resulted in a continuous increase in cytotoxic activity and viable cell numbers reaching a peak at day 4. If day 4 secondary MLC cells were separated by velocity sedimentation the cytolytic activity was associated with medium to large sized lymphocytes while the lytic activity generated during a subsequent 4 day restimulation was mainly associated with small lymphocytes. Thus day 4 secondary MLC cells could be separated on the basis of size differences into cytolytic cells and progenitors of cytolytic cells (*McDonald*, 1978). *Peck* and his collaborators stimulated purified T cells from mouse spleen with allogeneic spleen cells differing from the responders at the MHC (*Peck* et al., 1977a, b) or the MLs locus (*Peck* et al., 1977b). The primary MLCs (day 5) responder blasts were isolated 24 h after the peak of ^{3}H-TdR incorporation by 1*g*-velocity sedimentation and reexposed to the original and various other stimulator cells. The original stimulator cells induced a new wave of ^{3}H-TdR incorporation, while no ^{3}H-TdR incorporation was detected up to 60 h after exposure to spleen cells differing from the original stimulator cells in the MHC and/or the MLs locus. These data suggest, but do not prove, that the majority of the blasts expressed specificity for the original stimulator cells. The response to third party stimulator cells could have developed later than the secondary response to the original stimulator cells or was perhaps suppressed by the initially initiated secondary response. *Sondel* et al. (1975), for instance, detected in primary human MLC suppressor activity precluding the cytotoxic T cell response to third party stimulator cells.

δ) Nonfunctional Studies

A high degree of enrichment of specific lymphocytes at the peak of the response in MLCs was suggested by antigen and anti-idiotype binding studies (see Sect. IV. B. 4).

2. Reversion of Isolated MLC Blasts to Small Lymphocytes

a) Techniques

In many studies, the isolated blasts were allowed to revert back to small lymphocytes before restimulation. To this end, a variety of procedures were used:

1. Incubation of the blasts for 3 days (*Peck* et al., 1977a, b) or 10 days (*Wagner* and *Röllinghoff*, 1976) in the absence of other cells.

2. Incubation of the blasts with fresh irradiated stimulator cells for 14 days (*McDonald* et al., 1974b).

3. Incubation of blasts on macrophage or fibroblast feeder layers syngeneic to the responder cells for 4 days (*Andersson* and *Häyry*, 1973a, b, 1974, 1975) to 8 days (*Hollander* et al., 1974), following a procedure described earlier for reversion of mitogen-induced lymphoblasts (*Berke* et al., 1969; *Ginsburg* et al., 1971): *Andersson* and *Häyry* (1973a, 1974) recovered about 60% of blasts

(97% of the cells were blasts) after 5 days culture with feeder layers. Only 2% of the recovered cells were blasts and 95% were θ positive if nylon purified splenic T cells were used as responders (*Andersson* and *Häyry*, 1974). The time required for the reversion of the blasts to small lymphocytes appeared to depend on the plating density of the blasts on the feeder layers. The feeder layer cultures could not be extended beyond the 5th day because most of the cells died thereafter (*Andersson* and *Häyry*, 1974). Blasts and blast-derived small lymphocytes survived for much longer periods even in the absence of feeder cells but with appropriate amounts of mercaptoethanol in the culture medium (*McDonald* et al., 1974a; *Wagner* and *Röllinghoff*, 1976).

4. Injection of the blasts into T cell-deficient recipients syngeneic to the responder cells (*Andersson* and *Häyry*, 1973a; *Häyry* and *Andersson*, 1975a; *Häyry*, 1976) following the protocol of *Wilson* et al. (1972) and *Howard* and *Wilson* (1974), who injected in vitro activated rat TDL but omitted the blast fractionation step (see above): 36% of the blasts were trapped in the liver, 9% went to the spleen, and only 0.2%–0.6% to the lymph nodes (*Häyry* and *Andersson*, 1975a). Some injected cells, distinguishable from recipient cells by the T6T6 translocation marker, could be recovered from the recipient's spleen up to 60 days after transfer.

b) Characterization of Blast-Derived Lymphocytes

From all studies on blasts-derived lymphocytes (BDL) obtained by the procedures described, the following picture emerged. The recovered BDL were weakly cytotoxic for target cells syngeneic to the original stimulator cells and did not incorporate substantial amount of ^{3}H-TdR. They responded promptly upon reexposure to the original stimulator cells with proliferation and regeneration of cytotoxic activity. In some experiments, BDL did not respond at all to third party stimulator cells (*Peck* et al., 1977b), a somewhat surprising observation considering the strong cross-reactions of the proliferative responses of alloreactive T cells described by others (see below). In other cases, strong cross-reactivities of BDL were found at the level of restimulation and at the level of cytolysis, nonreactivity to responder type cells being the only and rather insufficient demonstration of immunologic specificity of the secondary response (*Häyry*, 1976).

3. Long-term Cultures

a) Techniques

Positive selection of alloreactive T cells was achieved most readily on the basis of selective survival of activated cells in long-term mixed leukocyte cultures. Mixtures of responder and stimulator cells were cultured for usually 10–20 days. During this prolonged primary MLC, the alloreactive T cells recognizing stimulator cell determinants were transformed into blast cells and reverted back to small nondividing lymphocytes with only weak cytotoxic activity (*McDonald* et al., 1974a, b). These small "secondary" lymphocytes responded, like the

blast-derived lymphocytes described above, upon reexposure to the original stimulator cells with proliferation and regeneration of cytotoxic activity. With successive restimulations, the responder cells could be further selected and propogated in tissue culture for several months (*McDonald* et al., 1974a; *Häyry* and *Andersson*, 1975b; *Häyry*, 1976; *Dennert* and *de Rose*, 1976) up to 1 year (*Watanabe* et al., 1977). Attempts to clone such cells are described in Section V.D. In the following, several aspects of long-term MLCs are described briefly to point out the difficulties and possible applications. For clarity, proliferative and cytotoxic T cell responses are considered separately although this is a somewhat artificial separation not made by most investigators.

b) Cytotoxic T Cells in Long-term Cultures

Detailed quantitative studies on cytotoxic T cells in long-term cultures were performed by *McDonald* et al. (1974a). Various times after initiation of cultures containing 5×10^6 C57Bl/6 spleen cells and 5×10^6 irradiated DBA/2 spleen cells viable cell counts were performed and cytotoxic activity on P815 tumor cells (DBA/2 origin) was determined in terms of lytic units (LU) per 10^6 cells. At day 4 almost half of the initially cultured responder cells were viable and gave 67 LU/10^6 cells. Viable cell recoveries and the cytotoxic activity decreased from day 6 on. At day 19 only 10% of input responder cells were viable and gave only 3 LU/10^6 cells. Within four cycles of restimulation in 20-day intervals, the number of viable cells decreased, and the values of LU increased slightly as determined 4 days after each restimulation. The data indicated therefore that the number of cytotoxic T cells remained constant during several cycles of restimulation (*McDonald* et al., 1974a).

However, subsequent studies have shown that cell proliferation in restimulated MLC is highly dependent on responding cell density. Restimulation of small numbers of primary MLC cells with an excess of stimulator cells resulted in a 15-fold increase in the number of viable cells and 2 000 fold increase in cytolytic activity (*Fitch* et al., 1975). A 500,000 fold increase in viable cell numbers and in cytolytic activity was observed when MLC cells were repeatedly restimulated at low responding cell density (*McDonald*, 1978)

Häyry and *Andersson* (1975b) restimulated CBA spleen cells repeatedly with mitomycin-treated DBA/2 spleen cells in 6-day intervals. The number of viable cells and maximal cytotoxicity did not change significantly within three cycles of restimulation, while the time required for regeneration of maximal cytotoxicity appeared to decrease gradually under these conditions. After the fourth to seventh cycle of restimulation, the cytotoxic but not the proliferative response was lost. Similar findings were described by *Watanabe* et al. (1977), while *Dennert* and *de Rose* (1976) maintained Balb/c anti-C57Bl/6 killer activity in some cultures that were continuously restimulated at 5–7-day intervals for 9 months.

The pattern of cross-killing of CTL generated in primary MLC and after several cycles of restimulation is identical (*McDonald*, 1978). However, at the induction level, the situation appears to be more complex. In mice CTL recognizing K and D region products of the stimulator cells could be restimulated by cells sharing only the I region with the primary stimulator cells (*Alter* et

al., 1976; *Bach* et al., 1977). Analogous results were obtained with human cells (*Zier* and *Bach*, 1975).

Tartof and *Fitch* (1977) were able to induce cytotoxic activity in 10–20-day-old primary MLC by stimulation with Con A or PHA. The cytotoxic activity of the positively selected cells was entirely restricted to target cells syngeneic with the primary stimulator cells. In contrast, in the experiments of *Dennert* and *de Rose* (1976), Con A and PHA inhibited proliferation and cytotoxic activity of cells from long-term MLCs possibly on the basis of nonspecific suicidal responder cell lysis mediated by Con A or PHA.

Continuous growth of human T-lymphocytes from the bone marrow (*Morgan* et al., 1976) and peripheral blood (*Ruscetti* et al., 1977) was maintained in conditioned medium obtained from PHA-stimulated normal human blood lymphocytes. For continuous proliferation, the conditioned medium had to be replaced every 4–5 days, and the cultures had to be readjusted frequently to relatively low cell densities. More recently, T cell growth factors obtained from Con A-stimulated mouse spleen cell cultures were used to maintain growth of T cells selected before in MLC (*Gillis* and *Smith*, 1977). High levels of cytotoxic activity were still found after 17 weeks of continuous growth. This technique will be very useful for cloning of specific T cells (Sect. V.D) and is currently being analyzed in more detail in several laboratories.

c) T Cell Proliferation in Long-term Cultures

Andersson and *Häyry* (1975) estimated the frequency of T cells responding in primary and secondary MLC to allogeneic stimulator cells by counting the number of cells which transformed in the presence of 10^{-2}–10^{-3} *M* hydroxyurea, a drug which allows blast transformation but inhibits DNA synthesis (*Sinclair*, 1965). In a primary response to DBA stimulator cells, 3%–5% CBA spleen T cells transformed into blasts. Restimulation at day 17 resulted in transformation of more than 90% of surviving cells, suggesting but by no means proving that the great majority of lymphocytes surviving in long-term cultures expressed specificity for the original stimulator cells.

Fathman et al. (1977) established a quantitative assay for proliferation (measured in terms of ^{3}H-TdR incorporation) by diluting responding cells obtained at day 14–21 from primary MLC. The rate of ^{3}H-TdR incorporation and cell proliferation as determined by viable cell counts was the same at day 2 and 3 after restimulation of 5×10^4–6.25×10^3 responder cells.

With the use of stimulator cells from recombinant congeneic mice, it was possible to map the genes coding for stimulating determinants in secondary MLR to the I region of the H-2 complex (*Fathman* et al., 1977). Often, strong proliferation was also observed after restimulation with cells of haplotypes entirely different from the haplotypes of the primary stimulator cells. The kinetics of the response to third party stimulator cells was like the kinetics of the response to original stimulator cells accelerated in comparison to primary responses.

The relative number of cells responding to various stimulator cells could be determined using small numbers of responder cells. Thus, about 50% of

A/J (H-2^a) cells primarily stimulated with C57Bl/6 (H-2^b) reacted to H-2^d and H-2^s and about 20% to H-2^f. Several possible reasons for this complex cross-stimulation pattern have been discussed by *Fathman* et al. (1977). Most likely, the proliferation induced by third party stimulator cells was not due to primary stimulation of surviving cells which were not stimulated before by the original stimulator cells, although survival of nonstimulated alloreactive T cells for 14 days in cultures has been observed (*McDonald* et al., 1974a; *Fradelizi* and *Dausset*, 1975).

4. Applications

a) Alloantigen-Binding T Cells

Nägy and his collaborators found that 4 days after in vitro stimulation of lymph node T cells with allogeneic stimulator cells, up to 44% of the blast cells were surface Ig-positive T cells. If nylon-purified T cells were used as responders, only 4% of T blasts had detectable amounts of surface Ig. Similar findings were described before with in vivo-activated T-TDL (Sect. III. C. 2). Alloantigen-activated T cells presumably bound stimulator antigens and subsequently B cell-derived alloantibodies directed against stimulator antigens. If T cells were activated in the absence of B cells, defined alloantisera could be used to identify specific alloantigen-binding T cells using an indirect immunofluorescence technique (*Nägy* et al., 1976a). Stimulator MHC determinants were detected in some cases on as many as 80% of the responder T blasts. Blasts activated in a response to allogeneic K or I region products were stained with antisera directed against either K or I region products (*Nägy* et al., 1976a, b). Cells binding K or I region products belonged to different T cell subclasses distinguished by anti-Ly antisera (*Nägy* et al., 1976b). Specific rebinding of alloantigen preparations to trypsinized T cell blasts indicated that the alloantigen receptors were synthesized by the T cell blasts themselves (*Elliott* et al., 1977).

b) Production and Use of Anti-idiotypic Antisera

Ramseier and *Lindemann* (1969) first described the production of antibodies to idiotypic determinants of recognition structures for alloantigen by immunization of F 1 hybrids with antialloantibodies of one parental strain to the other parental strain or by injection of mature lymphocytes of one parental strain (for review, see *Ramseier*, 1973). Anti-idiotypic antisera produced against alloantisera were found to bind to B-lymphocytes (*Binz* et al., 1974a), to inhibit binding of specific B cells and T cells to monolayers of the relevant allogeneic cells (*Binz* et al., 1974b), and to inhibit specific T cell functions (*Binz* et al., 1973).

Extensive studies were performed with anti-idiotypic antisera produced in F 1 hybrid rats using as immunogen highly purified peripheral T-lymphocytes of one parental strain. Such anti-idiotypic antibodies bound to the relevant IgG alloantibodies as well as to specific T- and B-lymphocytes and inhibited specific T cell functions in vivo or in vitro (for review, see *Binz* and *Wigzell*, 1977a, b). The anti-idiotypic binding to T cells was inhibitied with heavy chain

preparations of idiotype-bearing IgG alloantibodies (*Binz* and *Wigzell*, 1975a), and genetic analysis in rats indicated a linkage between idiotype and heavy chain allotype controlling genes (*Binz* et al., 1976).

More recently anti-idiotypic antisera were produced by immunization of animals with syngeneic T-lymphoblasts generated in MLCs against allogeneic cells (*Andersson, L.C.* et al., 1976). The sera from blast-immunized animals contained antibodies reacting with the relevant alloantibodies, with specifically activated T-lymphoblasts, and with molecules in concentrated culture supernatants from the MLCs in which the blasts were generated (*Andersson, L.C.* et al., 1976).

The procedure for production of anti-idiotypic antisera has been further developed by *Krammer* (1978). Highly purified lymph node T cells from mice were stimulated in MLCs against allogeneic stimulator cells, and the blasts were isolated at the peak of the proliferative response on a Ficoll density gradient. These isolated alloantigen-activated T cell blasts were used for the production of anti-idiotypic antisera in semiallogeneic crosses between the strains providing the responder and stimulator cells. It was found that a large proportion (up to 40%) of these blasts synthesized receptors binding both the relevant alloantigen preparations as well as anti-idiotypic antisera (*Krammer*, 1978). Genetic analysis suggested that the idiotypic determinants of the T cell receptors were controlled by genes in the heavy chain linkage group *and* the H-2 complex (*Krammer* and *Eichmann*, 1977).

c) Induction of Specific Unresponsiveness to Alloantigens

McKearn et al. (1974) first demonstrated that anti-idiotypic antibodies were produced in rats repeatedly immunized with alloantigens. *Binz* and *Wigzell* (1976a, b) developed a more efficient autoimmunization procedure by injecting Lewis rats with anti-DA receptor material isolated from Lewis serum or urine with anti-(Lewis-anti-DA) antibody-coupled immunoadsorbents. Subsequently, a more simple procedure was employed using as immunogen lymphoblasts generated in MLC. The blasts were isolated by 1-g velocity sedimentation and injected with complete Freund's adjuvant into syngeneic recipients (*Andersson, L.C.* et al., 1976, 1977). The injected mice were boosted twice with blasts in 3-week intervals. Spleen cells taken from 10 days up to more than 6 months after the last injection were specifically unresponsive to stimulator cells used in the primary selection culture. This effect could be demonstrated using various combinations of mice, rats, and outbred guinea pigs (*Andersson, L.C.* et al., 1977) and was shown to be mediated by auto-anti-idiotypic antibodies and auto-anti-idiotypic cytotoxic T cells (*Aguet* et al., 1978; *Binz* and *Wigzell*, 1978).

d) Identification of MLR-Stimulating Determinants With Selected Responder Cells

Resting alloreactive T-lymphocytes selected in long-term cultures were shown to proliferate upon reexposure to the original stimulator cells (Sect. IV. B 3.c). The secondary response was much stronger and faster than the primary response.

With small numbers of primed responder cells, a quantitative comparison of the response to different stimulator cells could be made thereby making it possible to map the stimulating determinants. With this technique, high cross-reactivity among MLR-stimulating determinants was found (*Fathman* et al., 1977), and MLR-stimulating determinants on F1 cells not present on either parental cells could be identified (*Fathman* and *Nabholz*, 1977). Human T cells positively selected in primary MLC were used to type cells for HLA-D determinants in short-term (24-h) assays (*Sheehy* et al., 1975; *Mawas* et al., 1975; *Hirschberg* et al., 1975; *Sasportes* et al., 1978a, b; *Nunez-Roldan* et al., 1978; *Wank* et al., 1978).

C. Positive Selection of T Cells Responsible for DTH Reactions to Conventional Antigens

1. Techniques

Several authors have demonstrated the importance of macrophage-associated antigen for the proliferative in vitro response of T cells from immunized donors to conventional (non-MHC) antigens (*Seeger* and *Oppenheim*, 1970; *Rosenstreich* and *Rosenthal*, 1973; *Shevach* and *Rosenthal*, 1973; *Paul* et al., 1977). *Lipsky* and *Rosenthal* (1975) found that more T-lymphocytes from immunized donors bound to macrophages pulsed with the immunizing antigen than to macrophages pulsed with an "irrelevant" antigen. Maximum binding was observed 24 h after incubation of antigen-pulsed macrophages with immune cells. These findings and the data on in vitro selection of alloreactive T cells described above prompted *Ben-Sasson* et al. (1975a) to develop an in vitro system for positive selection of T cells responsive to conventional antigens.

Peritoneal exudate lymphocytes from guinea pigs immunized 2 weeks to 2 months previously with various antigens in CFA in the footpads were passed through rayon wool columns (*Ben-Sasson* et al., 1975a). The effluent population highly enriched for T-lymphocytes was added to dishes containing adherent antigen-pulsed macrophages from nonimmunized syngeneic guinea pigs. Nonadherent cells were discarded 24 and 48 h later, and the macrophages with adherent lymphocytes were kept in tissue culture for various periods (usually for another 5 days). Seven days after initiation of the culture, T-lymphocytes were recovered that responded to the antigen used for pulsing the macrophages but only weakly to another antigen against which the responders had also been immunized. The cells discarded at 24 and 48 h showed a normal or only slightly reduced response to the macrophage-associated antigens, indicating the lack of a quantitative assay for antigen-responsive T cells and/or the failure to efficiently select specific T cells by binding to antigen-pulsed macrophages. However, several additional experiments suggested that both specific binding to antigen-pulsed macrophages and selective survival of proliferating cells contributed to the obtained selection of antigen-responsive T cells (*Ben-Sasson* et al., 1975b). Antigen-reactive T cells could be maintained for 2–3 weeks in tissue culture by weekly transfer to fresh antigen-pulsed macrophages. Generally, the antigen responsiveness disappeared by that time; a somewhat obscure antigen-independent prolifer-

ation could be maintained for at least 2 months in only a few cultures. More quantitative assay systems and an analysis of the assumed requirement for accessory cells (nonmacrophages) would be essential to determine the actual degree of purification of antigen-reactive T cells that can be obtained using the described procedures.

2. Applications: Studies on the Specificity of T Cell Receptors

T-lymphocytes positively selected in vitro by the technique of *Ben-Sasson* et al. (1975a) were used to investigate the nature of the binding of specific T-lymphocytes to antigen-pulsed macrophages. Binding of specifically selected T cells to antigen-pulsed macrophages was already detected 1 h after the cells were mixed (*Lipscomb* et al., 1977). The binding was shown to depend on pulsing the macrophages with the relevant antigen (*Ben-Sasson* et al., 1977) and was not inhibited in the presence of anti-guinea pig Ig (*Lipscomb* et al., 1977). The specific binding was not inhibited by a large excess of free or Sepharose-coupled antigen or by treatment of the antigen-pulsed macrophages with antiserum directed against the antigen (*Ben-Sasson* et al., 1977). Functional studies in vitro (*Rosenthal* and *Shevach*, 1973) and in vivo (*Vadas* et al., 1977) indicated that DTH T cells recognize conventional antigens in close association with MHC gene products. These findings were interpreted, like analogous findings in studies on cytotoxic T cells, to mean that T cells recognize new antigens determined by the native antigen and genes of the MHC. Recent experiments by *Zinkernagel* et al. (1978a, b) and *von Boehmer* et al. (1978a), however, suggest that T cells recognize conventional antigens and MHC products as distinct entities.

D. Positive Selection of Specific T Helper Cells for Humoral Immune Responses

Recently, a technique very similar to the one described in the previous Section was used for separation of specific T helper cells (*Swierkosz* et al., 1978). Peritoneal exudate cells from normal mice that adhered to culture trays were pulsed with SRC. Nylon wool-purified T cells from spleens of mice injected 4 days previously with SRC were added and the trays maintained at 37° C on a slowly rocking platform for 20–22 h. Nonadherent cells were then removed and macrophage-adherent cells recovered by mechanical means. The adherent fraction was further purified by passage through nylon wool columns to remove macrophages. F1 T cells incubated with SRC-pulsed parental macrophages collaborated more efficiently with B cells of the macrophage donor parent than with B cells of the other parent. Nonadherent cells were not depleted in helper activity, indicating that the T helper cell assay was not quantitative or that the observed effect was not due to physical separation of the two sets of F1 hybrid T cells recognizing SRC in association with either parental H-2 gene products.

E. Positive Selection of Specific B-Lymphocytes

No attempts have yet been made to select specific B-lymphocytes in tissue culture. Antigen-stimulated B-lymphocytes differentiate sooner or later into AFC, and to our knowledge, nothing is yet known about the generation of memory B cells in tissue culture. Experiments with mitogen-reactive B cells indicated that B cells grow continuously at low cell densities under conditions which do not favor differentiation into AFC (*Melchers* et al., 1975; *Andersson, J.* et al., 1977). B cell growth was dependent on the presence of mercaptoethanol, LPS, and a growth-supporting fetal calf serum (*Melchers* et al., 1975). All B cell "lines" were, however, lost after several weeks, possibly due to terminal differentiation into plasma cells, feedback inhibition, or the loss of an internal growth factor.

Premkumar-Reddy et al. (1976) described continuous culturing of B-lymphocytes from several strains of mice for more than 1 year in tissue culture flasks with growth-supporting surfaces that had been coated with rat tail collagen. Initially, spleen fragments were cultured. The growing cells were adherent B cells that were serially passaged after trypsinization into fresh flasks. The growth was independent of mercaptoethanol but most likely dependent on the B cell mitogenicity of the fetal calf serum used or the collagen surface. Alternatively, the cells were transformed by virus, although they failed to grow after intraperitoneal injection in syngeneic mice. For positive selection and propagation of specific normal B cells in tissue culture, more knowledge is required about the factors controlling growth and differentiation of B cells.

F. Selective Elimination of Antigen-Reactive B and T Cells

Puck and *Kao* (1967) eliminated proliferating hamster cells without affecting nonproliferating cells by β-emitting ^{3}H-thymidine (^{3}H-TdR), which can be incorporated in lethal amounts into DNA of mammalian cells, or by treatment with the thymidine analogue bromodeoxyuridine (BUdR), which can substitute about half of the thymidine in the DNA of dividing cells, resulting in a considerable increase in light and x-ray sensitivity of the proliferating cells (*Djordjevic* and *Szybalski*, 1960). Suicidal ^{3}H-TdR incorporation was first applied into immunology by *Dutton* and *Mishell* (1967) and BUdR and light treatment by *Zoschke* and *Bach* (1971a, b) as tools for selective killing of antigen-reactive lymphocytes in which DNA synthesis was induced by in vitro stimulation with antigen.

1. Techniques

a) Suicidal ^{3}H-Thymidine Incorporation

^{3}H-thymidine of high specific activity (5–20 Ci/mmol) is added to cultures to give a final activity of 5–15 μCi per milliliter of culture (Table 7). The cultures are pulsed at various time points depending on the response kinetics, usually for a period of 12–24 h. In control cultures, the hot pulse is blocked by addition

Table 7. Elimination of specific lymphocytes by a hot pulse with ^{3}H-TdR

Responder cells	Cells per ml culture	^{3}H-TdR (μCi/ml)	Spec. act. (Ci/mmol)	Hot pulse from-to	Inactivation of	References
Mouse spleen	1.5×10^6/ml	10	15	24–48 h	AFCP (T helper)	*Dutton* and *Mishell* (1967)
Mouse spleen	$10–1.5 \times 10^6$/ml	10	5	0–24 h 24–48 h	AFCP (i) AFCP (n+i) (not of T helper)	*Trowbridge* (1972)
Mouse spleen	12×10^6/ml	15	16	0–24 h	AFCP (i) AFCP (n+i) (not of T helper)	*Kettman* et al. (1973)
Human PBL	2×10^6/2 ml	5	18	48–66 h	Alloreact. T cells	*Salmon* et al. (1971)
Mouse lymph node	0.5×10^6/0.2 ml	3.5	?	48–72 h or 72–96 h	Alloreact. T cells	*Cantor* and *Jandinski* (1974)
Mouse spleen	25×10^6/5 ml	5	20	36–48 h	CTLP	*Peavy* and *Pierce* (1975)

From normal animals: n; from immunized animals: i.

of a large excess of cold thymidine. To terminate the hot pulse, the cells are washed and resuspended in medium containing an excess (100 μg/ml) of cold thymidine. To reestablish the original culture conditions, the cells should be resuspended in conditioned medium obtained from control cultures (*Trowbridge,* 1972), and the washed cells should be replaced in the original culture vessels, which should also be washed (*Peavy* and *Pierce*, 1975). After a hot pulse with ^{3}H-TdR, proliferative responses may be assessed with ^{14}C-thymidine (*Salmon* et al., 1971) or with ^{125}IUdR (*Cheers* et al., 1974).

b) Treatment With BUdR and Light

BUdR is added 48–72 h after initiation of the cultures or in 24-h intervals for 96 h (Table 8) to give a final concentration of 10^{-5}–10^{-6} *M* BUdR. Usually 24 h after BUdR addition, the cultures are exposed to light for 60–90 min or twice for 60 min (*Zoschke* and *Bach,* 1971a, b). During the exposure to light, an elavation of culture temperatures or a decrease in the CO_2 pressure should be avoided (*Rich* et al., 1972). Excess of BUdR may then be eliminated by washing the cultured cells (*Zoschke* and *Bach*, 1971a, b; *Janeway* and *Paul*, 1976). Control cultures are treated with either BUdR or light.

2. Applications

a) Elimination of Antigen-Reactive B Cells

Dutton and *Mishell* (1967) stimulated normal mouse spleen cells in vitro with burro erythrocytes (BRC). One day after the initiation of the culture, sheep erythrocytes (SRC) were added and ^{3}H-TdR which was "chased" 24 h later with cold thymidine. The hot pulse eliminated the PFC response to BRC almost completely but not the anti-SRC PFC response, indicating that normal antigen-reactive lymphocytes in this culture system do not synthesize large amounts of DNA within the first 24 h after contact with antigen and that different cells respond to different antigens.

Similarly, primary antihapten responses were eliminated by a 24–48 h hot pulse while a secondary anti-SRC response was eliminated by a hot pulse given after the first 24 h (*Kettman* et al., 1973; *Trowbridge,* 1972). SRC-specific helper activity was not affected by the hot pulse, indicating that antigen-specific B cells were eliminated in the cases described above (*Kettman* et al., 1973; *Trowbridge*, 1972). Radioresistance of the function of recently in vivo-activated T cells has been demonstrated (*Katz* et al., 1970; *Kettman* and *Dutton*, 1971; *Hamaoka* et al., 1972), although primary T cell responses in vivo require cell proliferation (*Mitchell* and *Miller*, 1968; *Sprent* et al., 1974).

b) Elimination of Alloreactive T Cells

Zoschke and *Bach* (1971a) attempted to eliminate from human PBL T cells proliferating in response to a particular type of allogeneic stimulator cell by treatment of MLC with BUdR and light. In some experiments, the secondary

Table 8. Elimination of specific lymphocytes by treatment with BUdR and light

Responder cells	Cells per ml culture	BUdR conc.	Added at h	Illumination for	Inactivation of	Reference
Human PBL	$0.4–0.8 \times 10^6$/2 ml	$10^{-6}-10^{-5}M$	72 (96)	2×60 min	DTH T cells	*Zoschke* and *Bach* (1970)
Human PBL	$0.25–2 \times 10^6$/8 ml	$10^{-5}M$ (final conc.)	24 (48, 96)	2×60 min	Alloreact. T cells	*Zoschke* and *Bach* (1971a)
Rat spleen	$2–4 \times 10^6$/2 ml	$2.5 \times 10^{-6}M$	72	$1 \times 60–90$ min	Alloreact. T cells	*Rich* et al. (1972)
Guinea pig T cells (PEL)	2×10^6/ml	$3 \times 10^{-6}M$	48	1×90 min	DTH T cells	*Janeway* and *Paul* (1976)
Guinea pig T cells (PEL)	6×10^6/3 ml	2 μg/ml	48	1×90 min	Alloreact. T cells	*Thomas* and *Shevach* (1977)

response to the original stimulator cells was significantly decreased while normal responses were obtained to third party stimulator cells. In other cases, no elimination of specific alloreactive cells and enhanced responses to third party stimulator cells were obtained.

In similar studies, *Salmon* et al. (1971) eliminated specific alloreactive cells from human PBL by a hot pulse with ^{3}H-TdR. *Rich* et al. (1972) eliminated specifically cells reactive to allogeneic cells in vitro and in vivo by treatment of MLC with BUdR and light. *Cheers* et al. (1974) found that addition of ^{3}H-TdR in high concentrations (10 μCi/ml) to cultures containing thoracic duct lymphocytes and irradiated stimulator cells for the period between 24 and 48 h inhibited DNA synthesis (as measured by ^{125}IUdR uptake). In contrast, ^{125}IUdR uptake of in vivo-activated T cells (T-TDL, Sect. III.C) was not affected by the presence of high ^{3}H-TdR concentrations during the period of 6–48 h of in vitro restimulation. This was interpreted to mean that T-TDL respond to restimulation by DNA synthesis without subsequent cell division (*Cheers* et al., 1974).

More recently, *Peavy* and *Pierce* (1975) used the hot pulse technique to eliminate specific cytotoxic murine T-lymphocytes. A 36–48 h hot pulse was sufficient to eliminate cytotoxic T cells specific for the initial stimulator cells but did not affect CTL responses to third party stimulator cells initiated after the hot pulse. We were unable to reproduce this finding using the same technique except that fresh original stimulator cells were added after the hot pulse in our experiments (*Haas*, unpublished data). *Cantor* and *Jandinski* (1974) also eliminated specific CTL in MLCs by a hot pulse between 24–72 h after initiation of the culture. Addition of cells, depleted of CTL by absorption on monolayers syngeneic to the stimulator cells, did not restore the cytotoxic response of pulsed cultures.

c) Elimination of Specific DTH T Cells

Zoschke and *Bach* (1970) stimulated human PBL in vitro with various antigens. The proliferative secondary response to one particular antigen was specifically eliminated by treatment of the primary culture with BUdR and light. More recently, *Janeway* and *Paul* (1976) studied the specificity of peritoneal exudate lymphocytes. Cells responding to one DNP conjugate were selectively destroyed by treatment with BUdR and light, and the capacity of the surviving cells to respond to other DNP conjugates was tested. These experiments suggested that the majority of the responsive T cells did not recognize only the hapten but also additional determinants of the carrier.

Thomas and *Shevach* (1977) eliminated alloreactive guinea pig T cells by treating mixtures of T cells and allogeneic macrophages with BUdR and light. The surviving cells responded to TNP-coupled allogeneic macrophages but not to allogeneic macrophages, and the cells stimulated initially to TNP-coupled allogeneic macrophages could be restimulated by TNP allogeneic macrophages but not TNP syngeneic macrophages. The conclusion that T cells can recognize antigens in association with allogeneic MHC products is difficult to reconcile with recent experiments by *Zinkernagel* et al. (1978a, b) and us (*von Boehmer*

et al., 1978a). Cross-reactivity between TNP-coupled and allogeneic cells (Sect. III.B.3.b) may explain the findings of *Thomas* and *Shevach* (1977).

Rosenthal et al. (1978) found that in vitro activation of pork insulin-immune 2×13 F1 guinea pig lymphocytes to insulin-bearing parental macrophages and subsequent exposure to BUdR and light decreased insulin responsiveness to insulin-bearing macrophages identical to those originally used for activation but not to insulin-bearing macrophages of the other parental strain. This showed that in F1 hybrid animals at least two distinct sets of insulin-reactive T cells exist. The interpretation was as suggested before (*Paul* et al., 1977) that immune response genes may operate at the level of antigen-presenting cells by selecting in a complex antigen the determinants recognized by immune T cells. We favour the idea that F1 hybrid animals have two sets of insulin-reactive T cells, each expressing two receptors, one for insulin and one for either parental histocompatibility antigens.

V. Isolation of Lymphocyte Clones

A. Introduction

Hemopoietic progenitor cells with high proliferative capacity were shown to form visible colonies in irradiated hosts or in semisolid media in tissue culture. Differentiation of pluripotent stem cells to lymphocytes could not be observed in these cloning systems, but similar techniques were employed to investigate antigen- or mitogen-induced growth and differentiation of mature lymphocytes. The progeny of single lymphocytes was detected in restricted areas of irradiated host spleens (Sect. V.B.1) or as clusters or colonies in semisolid media in tissue culture (Sect. V.D). A restricted mobility of the proliferating cells is essential for these techniques, and the probability that cells of different clones are separated from each other increases with decreasing numbers of cells transferred or cultured. Transfer of very few cells into many recipients (Sect. V.B.2) or liquid cultures (Sect. V.C) permits the isolation of clones of lymphocytes of one particular specificity in a few recipients or cultures.

B. Isolation of Lymphocyte Clones in Vivo

1. Isolation of Lymphocyte Clones in Spleen Fragments of Irradiated Recipients Injected With Small Numbers of Syngeneic Cells

Till and *McCulloch* (1961) first described an in vivo cloning system for hemopoietic stem cells present in bone marrow of mice. Visible colonies consisting of erythroid and myeloid but not lymphoid cells were found in the spleens of irradiated recipients transferred with syngeneic bone marrow cells (*Wu* et al., 1967). Thus, spleen colony techniques do not allow the study of differentiation of stem cells to lymphocytes. However, similar techniques were developed that allow the study of the antigen-dependent differentiation of mature lymphocytes into executive cells such as AFC.

In 1965, the splenic focus assay or hemolytic focus assay was developed by *Kennedy* et al. (1965, 1966) and *Playfair* et al. (1965) to detect and analyze the progeny of single AFCP. The spleens of irradiated mice transferred with spleen cells and immunized with SRC were cut into many slices, which were placed on a layer of agar-containing SRC. Complement-dependent lysis of SRC was observed around some slices, indicating the presence of foci of anti-SRC AFC. The number of hemolytic foci was directly proportional to the number of spleen cells transferred, indicating that one of at least two types of transferred cells required for focus formation (*Gregory* and *Lathja*, 1968; *Mitchell* and *Miller*, 1968) was limiting. A modification of the hemolytic focus assay was based on the localized immobilization of flaggelated bacteria to detect foci of AFC (*Armstrong* and *Diener*, 1969). In most studies, the confluence of foci caused considerable problems, particularly if more than 4×10^6 cells were transferred (*Armstrong* and *Diener*, 1969; *Cunningham,* 1969a). The difficulties were reduced by using conventional plaque techniques for detection of individual AFC in suspensions obtained from small pieces of spleens (*Celada* and *Wigzell*, 1966a, b; *Nakano* and *Braun*, 1966; *Cunningham*, 1969a, b). *Klinman* and collaborators (*Klinman*, 1969; *Klinman*, 1971a, b; *Klinman* and *Aschinazi*, 1971) attempted to prevent the in vivo mixing of cells derived from different precursors by in vitro stimulation of individually sliced spleen fragments obtained from irradiated mice that were injected with small numbers of normal lymphocytes 16 h before (Sect. V.C.1).

2. Isolation of Lymphocyte Clones in Spleens of Irradiated Mice Injected With Limiting Numbers of Syngeneic Cells

Adoptive immune responses of limiting numbers of lymphocytes in syngeneic irradiated recipients have been studied frequently to enumerate and to characterize so-called immunocompetent units (IU) or antigen-sensitive units (ASU), i.e., the minimal combination of interacting cells required for the response to one particular antigen (*Brown* et al., 1966; *Bosma* et al., 1967, 1968; *Shearer* et al., 1969; *Bosma* and *Weiler*, 1970; *Miller* and *Cudkowicz*, 1970; *Orsini* and *Cudkowicz*, 1971; *Mozes* et al., 1970; *Shearer* et al., 1971; *Möller* and *Michael*, 1971; *Groves* and *Christian*, 1973). Some authors used Millipore diffusion chambers that were implanted in hosts (*Groves* et al., 1970; *Goodman* et al., 1972).

Askonas et al. (1970) used sequential spleen cell transfers in syngeneic recipients as a tool for selection of the progeny of a single antigen-reactive B cell; $1–5 \times 10^6$ spleen cells from immunized mice were transferred intravenously into a series of irradiated (600 rad) recipients together with antigen. The cloning efficiency and the clone size was considerably enhanced if antigen-pulsed macrophages were injected together with the primed cell population (*Askonas* and *Roelants*, 1974). Mice were selected as spleen donors for a second transfer on the basis of restricted isoelectric spectra of specific serum antibodies; $4–5 \times 10^6$ spleen cells of the selected mice were serially transferred into new irradiated recipients, which also received antigen (*Askonas* et al., 1970). However,

in a large number of studies (*Askonas* et al., 1972, 1976; *Askonas* and *Williamson*, 1972a, b; *North* and *Askonas*, 1974; *McMichael* and *Williamson*, 1974; *McMichael* and *Willcox*, 1975), no evidence was provided that specific B cell clones were separated by this technique from other B cells of the same specificity. In some cases, it was clear that the production of antibody with restricted heterogeneity was due to clonal dominance rather than to the isolation of single AFCP and their progeny. If the level of homogeneous antibody dropped below a critical threshold (*Askonas* and *Williamson*, 1972b) or if apparently cloned cells were treated with radioactively labeled antigen (*McMichael* and Willcox, 1975), new clones came up. The fact that T cell-dependent antigens were used and often carrier-primed helper cells were added makes the interpretation of these studies rather difficult. Sequential spleen cell transfers were also used by *Briles* and *Krause* (1972) and *Eichmann* (1972, 1973) to produce antistreptococcal antibodies with one particular idiotype.

3. Applications

a) Ig Expression in B Cell Clones

Clones of anti-SRC and anti-CRC (*Celada* and *Wigzell*, 1966a) or anti-SRC and antigoat red cell AFC (*Cunningham*, 1969b) were found to be independent of each other in the hemolytic focus assay. Antibodies produced by single spleen foci were at least in some cases homogeneous with respect to electrophoretic mobility (*Luzzati* et al., 1970; *North* and *Feinstein*, 1973). AFC-producing IgM and IgG were found in single foci consistently (*Sterzl* and *Nordin*, 1971; *Campbell*, 1971) or only occassionally (*Cunningham*, 1969b; *Celada* and *Wigzell*, 1966b; *Papermaster*, 1967). Similar findings were made using mice injected with limiting cell numbers. ASUs to SRC and CRC were separated, the limiting cells being B-lymphocytes (*Miller* and *Cudkowicz*, 1970; *Shearer* et al., 1971). ASUs to SRC also appeared to be restricted to produce either IgM or IgG, and this Ig class restriction was determined by B cells (*Cudkowicz* et al., 1969; *Shearer* and *Cudkowicz*, 1969; *Miller* and *Cudkowicz*, 1970; *Orsini* and *Cudkowicz*, 1971). ASUs to poly-D-alanine of F1 hybrid spleen cells were restricted to produce IgG of only either of the two parental allotypes, which were found simultaneously in recipients of larger numbers of hybrid cells (*Bosma* and *Weiler*, 1970).

b) Frequency of Antigen-Reactive Lymphocytes

The hemolytic focus technique and the technique of limiting cell dilutions in adoptive transfer experiments were used to estimate the frequency of antigen-reactive B cells (for reviews, see *Halsall* and *Makinodan*, 1974; *Lefkovits*, 1974) or T cells (*Shearer* et al., 1969; *Groves* et al., 1970; *Vann* and *Campbell*, 1970) in normal and immunized mice. A detailed description of these studies is avoided

here because the value of these estimates is rather limited for the following reasons:

1. The observed frequencies of antigen-reactive lymphocytes depend on the homing efficiency of transferred cells, which cannot be determined precisely, estimates ranging from 1% (*Askonas* et al., 1972) to 15% (*Kennedy* et al., 1966).

2. In most studies, it is not clear whether AFCP or T helper cells were limiting.

3. The observed frequency of lymphocytes responding to a particular antigen mainly reflects the experimental conditions rather than the frequency of potential precursors.

However, in this context, some earlier studies should be mentioned. When graded numbers of large peripheral blood lymphocytes (*Szenberg* and *Warner*, 1961) or small lymphocytes (*Simons* and *Fowler*, 1966) obtained from fowls were inoculated on the chorioallantoic membrane of chick embryos, it was noted that a large proportion of these cells produced easily visible, opaque foci (indicating a GvH reaction). A high frequency of PBL (1–2%) specific for one foreign allele of the B locus was also observed in experiments in which the spleen weights were determined in chicken embryos injected intravenously with PBL dilutions from adult fowls (*Simonsen*, 1967; *Nisbet* et al., 1969).

c) T-B Collaboration in Humoral Immune Responses

Some models for the mechanism of cell-cell interactions in anti-SRC responses were deduced from the comparison of the frequencies of ASUs to SRC by both limiting dilution analysis and the hemolytic focus assay (*Groves* and *Christian*, 1973). *North* and *Feinstein* (1973) transferred spleen cells from NIP-BSA-primed mice into syngeneic irradiated recipients that were challenged with NIP-BSA. Single hemolytic foci detected in cultured spleen fragments were found to produce electrophoretically homogenous anti-NIP antibodies. If known mixtures of NIP-ovalbumin (NIP-OVA)-primed and BSA-primed cells were transferred and if the number of hemolytic foci was limited by the number of carrier-primed donor cells and hapten-primed B cells were given in excess, a surprisingly low increase in the heterogeneity of antihapten antibodies was observed. These findings suggested that either the development of one particular clone of NIP-specific B cells did not allow other NIP-specific AFCP in the vicinity of this "dominating" clone to proliferate and/or differentiate into AFC or alternatively that the T helper cells required for focus formation was restricted to collaborate only with a single AFCP. More recent experiments made the first possibility unlikely (*Sullman* and *Feinstein*, 1977). An excess of B cells primed to two different haptens (H1 and H2) was transferred together with limiting numbers of T cells primed to one carrier (C1). The incidence of foci specific for each hapten was 21% after challenge with H1–C1 plus H2–C1. The same incidence of monospecific foci was found if in addition cells primed to a second carrier (C2) were injected followed by challenge with H1–C1 and H2–C2. This was interpreted to indicate that one T helper cell was restricted to help one AFCP only.

C. Isolation of Lymphocyte Clones in Vitro

Various approaches were used to induce in vitro an immune response of single lymphocytes of one particular specificity by limiting dilution. In the simplest case, responses to antigens or mitogens were studied that do not require cell-cell interactions. More complex immune responses in limiting dilution cultures require often rather artificial experimental protocols to provide optimal conditions for the growth and differentiation of the limiting cell type. If specific or nonspecific helper cells, accessory cells, or feeder cells are added, they must be devoid of the limiting cell under study. A detailed description of tissue culture techniques is beyond the scope of this article, but several approaches to limiting dilution cultures are described briefly.

1. Spleen Fragment Culture Techniques

Klinman developed an in vitro splenic focus assay for isolation of antigen-stimulated B cell clones (*Klinman*, 1969, 1971a, b; *Klinman* and *Aschinazi*, 1971). Small numbers (8×10^5) of spleen cells from mice immunized several months before with DNP-hemocyanin were injected intravenously into syngeneic recipients that were lethally irradiated (650–900 rad) 1 day before. The spleens of the recipient mice were sliced 1 day after transfer into 1.0–1.2-mm cubes (about 30–50 fragments per spleen), and the fragments were cultured according to the method described by *Globerson* and *Auerbach* (1965) in tubes containing culture medium without mercaptoethanol. The fragment cultures were incubated at 37° C in a moist atmosphere of 93% O_2 and 7% CO_2 and the medium was changed every 2–3 days. The fragments were stimulated by addition of antigen to the culture fluid. Maximal stimulation was obtained by adding the antigen 24–48 h after the initiation of the culture (*Klinman* and *Aschinazi*, 1971). Antibody production by individual fragments was measured by a radioimmunoassay carried out on culture fluids for several weeks after stimulation with antigen. The number of antibody-producing fragments was directly proportional to the number of injected cells (*Klinman* and *Aschinazi*, 1971; *Press* and *Klinman*, 1973b). The cloning efficiency of the cell transfer and fragment cultures was estimated to be 3%–4% (*Klinman* et al., 1975b).

Antibody-producing fragments were obtained only from recipients injected with cells from immunized mice. Fragments from recipients injected with as many as 4×10^7 normal spleen cells gave no antibody response in the organ culture system (*Klinman* and *Aschinazi*, 1971). However, primary antihapten AFC derived from single precursor cells were detected in spleen fragments of carrier-primed, irradiated recipients that had been injected with cells from adult mice (*Klinman*, 1972; *Klinman* and *Doughty*, 1973) or with cells from neonatal mice (*Press* and *Klinman*, 1973a; *Klinman* and *Press*, 1975a, b, c; *Klinman* et al., 1976a, b). Spleen fragment cultures were also used in attempts to produce homogenous antibodies against influenza virus (*Gerhard* et al., 1975; *Gerhard*, 1976), β-galactosidase (*Macario* et al., 1972), murine tumor cells (*Segal* and *Klinman*, 1976), and human tumor cells and cell lines (*Levy* and *Dilley*, 1977; *Lampson* et al., 1977).

2. Suspension Culture Techniques

a) Isolation of B Cell Clones

Osoba (1969) first studied the AFC response to heterologous erythrocytes in Marbrook culture vessels (*Marbrook*, 1967) containing graded numbers of normal spleen cells and a large excess of irradiated syngeneic spleen cells to provide optimal cell densities. The response to two different antigens segregated into different cultures if the number of normal spleen cells was limiting. Irradiated "filler" cells had previously been used for a long time to increase the cloning efficiency of tumor cells in tissue culture (*Puck* and *Marcus*, 1955). *Halsall* and *Makinodan* (1974) also used the Marbrook culture system and found that red blood cells as fillers could not provide optimal conditions. These authors attempted, therefore, to culture small numbers of spleen cells at optimal density by reducing the surface area of the Markbrook chambers. Maximal responses were obtained in cultures containing 3×10^5 cells per mm^2 surface area. However, this technique does not enable one to culture less than 10^6 spleen cells per culture at optimal cell density.

Hunter and *Kettmann* (1974) cultured mouse spleen cells at limiting cell concentrations in plastic Petri dishes as described by *Mishell* and *Dutton* (1967). The AFC response of low numbers of spleen cells to various antigens was facilitated by addition of mercaptoethanol to the culture medium. T helper cells were limiting in a anti-SRC AFC response when normal spleen cells were cultured. The spleen cells from thymectomized and bone marrow reconstituted mice (AT × BM) gave very low responses to SRC or TNP-SRC, but the frequency of responding AFCP as well as their clone size was considerably enhanced by addition of irradiated SRC-primed spleen cells or allogeneic MLC supernatants. Allogeneic factors also enhanced the clone size of B cells responding to T-independent antigens.

Lefkovits (1972) developed a microculture system in which very small portions (10 µl) of a cell suspension at optimal density (2×10^7/ml) were dispensed with a multisyringe dispenser in the small flat bottom wells of plastic tissue culture trays. To study B cell responses to a T cell-dependent antigen (SRC), $0.2\text{–}1.8 \times 10^5$ spleen cells from nude mice were cultured with 2×10^4 allogeneic spleen cells as a source of nonspecific T cell help (*Lefkovits*, 1973) and irradiated spleen cells from nude mice to adjust the cell density ((*Quintans* and *Lefkovits*, 1973). In subsequent studies, mitogens were used in addition to antigen for stimulation (*Quintans* and *Lefkovits*, 1974a, b, c) and allogeneic T cells were replaced by purified antigen-activated T cells (*Vann* and *Dotson*, 1974; *Waldman* et al., 1975a) or a variety of specific and nonspecific T cell factors (*Vann* and *Dotson*, 1974; *Lefkovits* et al., 1975; *Waldmann*, 1977). The response of limiting numbers of spleen cells to a T-independent antigen (heat-killed pneumocci) did not require irradiated filler cells or allogeneic T cell factors (*Cosenza* et al., 1975; *Quintans* and *Cosenza*, 1976).

Jonard and *Panijel* (1973) also cultured small numbers of cells in microcultures (10 µl/culture). Normal spleen cells inactivated with an antibiotic (U9361) were used as filler cells. *Marbrook* and *Haskill* (1974) cultured the cells in an array of small dimples which were molded in the surface of a porous polyacryl-

amide vessel. This technique may be regarded as a limiting cell dilution culture under conditions of optimal cell density at least with regard to diffusible conditioning factors. The actual cell density in each well was additionally increased by syngeneic irradiated spleen cells or thymus cells. The apparently T cell-independent fraction of anti-SRC AFCP obtained in the light density fraction of spleen cells was used to study the antigen-induced generation of B cell clones. LPS-induced anti-SRC AFC responses of normal spleen cells were studied in this system by *Pilarski* and *Cunningham* (1974).

Nossal and his collaborators developed a microculture system primarily to study B cell responses to haptens coupled to the T-independent carrier polymerized flagellin (*Pike*, 1975; *Nossal* and *Pike*, 1976; *Stocker*, 1976, 1977a, b). Limiting numbers of spleen cells were cultured in 0.2 ml in flat-bottomed microtest II tissue culture plates (System Cook, Greiner, Germany). In most experiments, 2×10^6 syngeneic thymus cells were added per well to provide optimal cell densities (*Stocker*, 1976). The unfractionated thymus cells gave a low but significant background response, which could be eliminated, however, by pretreating the thymus cells with anti-Ia serum and complement (*Nossal* and *Pike*, 1978). Several other kinds of treatment of thymus cells (x-irradiation, mitomycin treatment, nylon wool purification, density cut separation) reduced background PFC responses but also support capacity of the filler cells (*Nossal* and *Pike*, 1978). In this system, the AFC responses of as few as 1–50 purified B cells could be studied.

Andersson, J. et al. (1976, 1977a, b, c) studied the in vitro response of limiting numbers of normal spleen cells to the B cell mitogen LPS in a variety of different culture vessels. The response to LPS was monitored by a plaque technique that allows detection of all Ig-secreting cells using protein A-coated SRC and Ig-specific antibodies (*Gronowicz* et al., 1976). The response of small numbers of spleen cells was increased slightly (about tenfold) by addition of a large excess of SRC as filler cells. A considerable increase in the frequency of LPS responding cells was found when nonirradiated, syngeneic, or allogeneic purified thymus T cells were added. In the presence of 2×10^6 thymus cells per milliliter, more than 100 IgM AFC were generated within 5 days in cultures containing initially an average of only six spleen cells. Limiting dilution analysis showed that one out of three B cells in normal spleens proliferated and differentiated into IgM AFC. The cloning efficiency of various lymphoid cell lines was also increased in the presence of appropriate numbers of thymus filler cells to near 100% (*Andersson, J.* et al., 1976; *Lernhardt* et al., 1978). Addition of filler cells to the limiting dilution cultures could be avoided if the medium and/or the culture vessels were conditioned by a 24-h preincubation with thymus cells (*Andersson, J. et al.*, 1977c). Recently, *Wabl* et al. (1978) described a technique that allowed culturing single LPS-induced B cell blasts in Terasaki wells over a feeder layer of LPS-stimulated spleen cells in 0.3% agar. Up to 200 cells were generated from single blasts within 4–5 days.

b) Isolation of Alloreactive T Cell Clones

Teh et al. (1977a, b, c) developed a microculture system for segregation of cytotoxic T-lymphocyte precursors and their progeny. Limiting numbers of nor-

mal RNA nu/+ lymph node cells (10^2–2×10^3) were cultured together with 2×10^5 RNC nu/nu spleen cells and 2×10^5 irradiated stimulator cells in 0.1 ml in V-bottom microtiter trays (*Teh* et al., 1977a). In some experiments, stimulator cells were replaced by T cell mitogens (*Teh* et al., 1977b). The nu/nu spleen cells that did not develop cytotoxic T cells were required as a source of apparently specific non-T cells (*Schilling* et al., 1976). Cell separation studies suggested that the synergistic activity of nu/nu spleen cells was the result of two subpopulations of cells with a precursor-progeny relationship (*Miller* et al., 1977a). Accessory cells were limiting in cytotoxic T cell responses of dilutions of nu/+ lymph node cells (*Teh* et al., 1977a; *Miller* et al., 1977b). After 7 days, each well was tested for cytotoxic activity on small numbers of different highly ^{51}Cr-labeled target cells. A very similar system was used by *Fischer-Lindahl* and *Wilson* (1977a, b). These authors cultured limiting numbers of responder cells without accessory cells but with a large excess of irradiated stimulator cells. More recently, *Teh* et al. (1977c) succeeded in restimulating T cell clones generated in limiting dilution cultures. Seven days after initiation of the microcultures, half of the cells of each well were tested for cytotoxicity on two different target cells. The remaining cells were cultured for a further 7 days, then restimulated and tested for cytotoxicity on the same target cells 3–4 days later. For restimulation, addition of fresh nu/nu spleen cells was not required.

Watanabe et al. (1977) studied mitogen-induced growth of T-lymphocytes in limiting dilution cultures. Small numbers of T cells could be stimulated to generate cytotoxic T cells only if syngeneic nude spleen cells were added in excess. Using these conditions, CTL responses were obtained with as few as 100 normal lymph node cells. The polyacrylamide dimple culture system described above was also used for studies on the development of cytotoxic T cells in cultures containing limiting numbers of spleen cells and semiallogeneic stimulator cells (*Skinner* and *Marbrook*, 1976; *Ching* et al., 1977a) or in the absence of stimulator cells (*Ching* et al., 1977b, c, d,).

c) Isolation of T Helper Cells

Isolated T helper cells were studied in 1 ml or 0.1 ml cultures (*Marrack* et al., 1974; *Marrack* and *Kappler*, 1975) or in 10 µl microcultures (*Waldmann* et al., 1975a, 1976a, b). Limiting dilutions of T helper cells were added to an excess of B cells. The source of B cells were spleen cells from adult thymectomized, irradiated, and bone marrow reconstituted mice (AT × BM) (*Marrack* et al., 1974; *Waldmann* et al., 1975a) or anti-θ and complement-treated spleen cells from immunized mice (*Marrack* et al., 1974; *Marrack* and *Kappler*, 1975; *Waldmann* et al., 1975a, 1976a, b). The source of T cells were spleen from irradiated mice injected 7 days before with 10^8 thymus cells and antigen (*Waldmann* et al., 1975a), spleen cells from recently primed mice (*Marrack* et al., 1974; *Marrack* and *Kappler*, 1975; *Waldmann* et al., 1976a), or from long-term primed mice (*Waldmann* et al., 1975a, 1976a, b). In some experiments, these cells were irradiated and passed through nylon wool columns to prevent subsequent cell divisions and to inactivate B cells (*Waldmann* et al., 1976a, b). Controls were included to demonstrate that all cultures contained responsive B cells when

nonlimiting numbers of helper cells were added (*Marrack* and *Kappler*, 1975), when allogeneic (*Marrack* and *Kappler*, 1975) or antigen-induced helper factors (*Lefkovits* et al., 1975) were added, or when T-independent antigens were used (*Marrack* and *Kappler*, 1975; *Waldmann* et al., 1976a). All these studies were concerned with the in vitro action of in vivo-primed and in vitro-restimulated T helper cells.

3. Semisolid Media Culture Techniques

More than 10 years ago, *Pluznik* and *Sachs* (1965), *Ichikawa* et al. (1966), and *Bradley* and *Metcalf* (1966) demonstrated proliferation of hemopoietic progenitor cells and differentiation in neutrophils and macrophages in semisolid media in tissue culture. These colonies resembled colonies obtained in the spleens of irradiated recipients (*Wu* et al., 1968). Subsequently, colonies of other cell types were obtained such as erythropoietic cells (*Stephenson* et al., 1971; *Gregory* et al., 1973; *Iscove* and *Seiber*, 1975), eosinophilic-like granulocytes (*Iscove* et al., 1971; *Chervenick* and *Boggs*, 1971; *Metcalf* et al., 1974) or megakaryocytes (*Metcalf* et al., 1975c). Recently, mixed colonies were obtained from single multipotential stem cells (*Johnson* and *Metcalf*, 1977). Differentiation of stem cells to lymphoid cells has not yet been observed. Clonal growth in semisolid media has been obtained with some tissue culture-adapted plasmacytoma cell lines (*Coffino* and *Scharff*, 1971; *Cotton* et al., 1973; *Metcalf*, 1973, 1974) and hybrid cell lines (*Köhler* and *Milstein*, 1975, 1976). Conditions shown to enhance colony formation of plasmacytomas (*Metcalf*, 1974), particularly addition of mercaptoethanol to the culture medium, were recently applied with success to obtain growth of colonies from normal lymphocytes in semisolid media. In the following, the mitogen-dependent colony formation of mature B- and T-lymphocytes and preliminary attempts to clone alloantigen-specific T cells are described briefly.

a) B Cell Colonies

Metcalf and his colleagues in the Walter and Eliza Hall Institute in Melbourne were the first to demonstrate B-lymphocyte colony formation in semisolid medium (*Metcalf* et al., 1975a, b). Lymphoid cell suspensions were suspended in agar (final concentration 0.3%) in Dulbecco's modified Eagle's medium (DMEM) supplemented with asparaginase, DEAE dextran, antibiotics, bicarbonate, unheated fetal calf serum, and mercaptoethanol. The cell suspensions in agar medium were pipetted in 1-ml portions into 35-mm plastic Petri dishes. Cultures containing 10^5–10^6 lymph nodes or spleen cells formed, within 4–5 days, lymphocyte clusters and colonies that were not observed in the absence of mercaptoethanol.

B-lymphocyte clusters and colonies were obtained from cells derived from bone marrow, lymph nodes, spleen, Peyer's patches, blood, peritoneal and pleural cavity, and thoracic duct lymphocytes of adult mice (*Metcalf* et al., 1975b)

and also from 17-day-old fetal liver, spleen, bone marrow, and peripheral blood (*Johnson* et al., 1976). The thymus was the only lymphoid organ in which very few if any colony-forming cells were found. The frequencies of colony-forming cells in these organs, obtained under optimal conditions (see above), varied between 10^{-3} and 10^{-2}. The cells giving rise to colonies were shown to be θ negative, Fc receptor positive, radiation and cortisone sensitive, nonadherent small lymphocytes with a buyont density similar to that of normal B-lymphocytes (*Metcalf* et al., 1976). Spleen cells from nu/nu mice (*Metcalf* et al., 1976) and hapten-gelatin binding B-lymphocytes (*Metcalf* et al., 1975b) also gave rise to colonies. Indirect evidence suggested that the cells that proliferated in agar medium were surface IgM and IgD positive and expressed Ia antigens (*Kincade* and *Ralph*, 1976; *Scott* et al., 1978) and may belong to the T cell-independent subclass of B cells (*Kincade*, 1977). Thus, most cells forming colonies of B-lymphocytes were themselves B-lymphocytes. B cell colony formation resembles in many respects clonal growth of B-lymphocytes in liquid cultures but is still much less efficient (*Andersson, J.* et al., 1976).

B-lymphocyte colony formation was shown to depend on mitogenic stimulation of B-lymphocytes by carbohydrate constituents of common agar (*Kincade* et al., 1976). The use of other known B cell mitogens such as bacterial lipopolysaccharide (LPS) allowed cluster and colony formation in low cell density cultures (*Metcalf*, 1976). In the presence of 10–20 μg LPS, 25,000 C57Bl/6 spleen cells formed approximately 250 colonies consisting of 50–2000 cells. The effect of LPS was shown to be at least in part due to direct stimulation of colony-forming cells (*Metcalf*, 1976).

Colony formation in agar was enhanced, particularly if small numbers of cells were cultured, by addition of 10^6 thymus cells, which themselves did not give rise to colonies or 10^6-irradiated spleen cells and by addition of 0.1 ml 10% washed SRC (*Metcalf* et al., 1975b). Adherent cells present in normal spleen and peritoneal exudate from irradiated mice were more efficient than nonadherent spleen cells to enhance colony formation (*Metcalf* et al., 1976).

Kurland et al. (1977) employed a two-layer soft agar culture system which consisted of 1 ml 0.3% agar top layer containing spleen or lymph node cells at low cell density and a 1 ml 0.5% agar bottom layer containing adherent macrophages. Colony formation in the top layer was enhanced or inhibited depending on the number of macrophages in the bottom layer. LPS and SRC, which appeared to stimulate colony-forming cells at least in part, were also able in this system to directly modify the release of enhancing or inhibitory factors by macrophages (*Kurland* et al., 1977).

b) T Cell Colonies

Clonal growth of PHA-stimulated human lymphocytes in soft agar was first demonstrated by *Rozenszajn* et al. (1975). Single colonies were composed of up to 500 T-lymphocytes. Presensitization of peripheral blood lymphocytes with PHA in short-term liquid cultures and the continuous presence of PHA in

the soft agar cultures was essential. Subsequently, similar findings were described by several other authors (*Wilson* and *Dalton*, 1976; *Riou* et al., 1976; *Zeevi* et al., 1977; *Fibach* et al., 1976; *Maurer* et al., 1977). In all these studies, the plating efficiences of human peripheral blood cells was never higher than 0.1%–0.3%. Recently, a more efficient technique was described by *Claësson* et al. (1977a). Mononuclear cells were separated from the blood of healthy humans by centrifugation through Ficoll-Isopaque (*Böyum*, 1968): 10^4–5×10^4 viable mononuclear cells were plated directly in 5 ml agar medium in 35-mm plastic dishes (one-step procedure) or 2.5×10^3–25×10^3 cells were plated after prestimulation for 18 h with PHA in liquid cultures (two-step procedure). The medium contained 0.45% agar, serum, mercaptoethanol, washed erythrocytes, and PHA. The frequency of cells forming colonies of more than 50 cells was 1 in 100 plated cells in the one-step procedure and 1 in 20 presensitized cells. The colony-forming cells were small, noncycling lymphocytes that formed, like their progeny E rosettes, a marker for human T cells.

Colony formation was dependent on the presence of the mitogen and was enhanced by soluble, pronase-sensitive factors released from adherent cells (*Claësson* et al., 1977b). An excess of factors released from adherent cells may inhibit colony formation of human T cells, while enhancement of colony formation was obtained by addition of culture supernatants from PHA-stimulated T-lymphocytes (*Zeevi* et al., 1977) or certain lymphoblastoid cell lines (*Galbraith* et al., 1977). Colonies were also obtained from mouse T-lymphocytes in a two-layer soft agar culture system (*Sredni* et al., 1976). However, only 0.01% of mitogen prestimulated lymph node cells formed colonies consisting of 50–1000 θ-antigen-bearing lymphocytes. *Watanabe* et al. (1977) obtained Con A-induced colonies from purified mouse T cells in gelatin, methylcellulose, or agar as supporting media, which was also supplemented with serum and mercaptoethanol. Colony formation was strictly dependent on the presence of Con A. The frequency of colonies was markedly enhanced if the T cells were presensitized in high cell density cultures and if filler cells were included in a bottom agar layer. However, the frequency of colony-forming cells in purified lymph node T cells was still only about $1\text{–}2 \times 10^{-2}$.

The same system was also used in attempts to clone alloreactive T-lymphocytes which were positively selected in long-term cultures by repeated restimulation (*Watanabe* et al., 1977; *Fathman* and *Hengartner*, 1978). Two days after the fifth restimulation, responder cells were seeded with a large excess of irradiated stimulator cells in soft agar (0.25%) over a base layer of 0.5% agar. Colonies were picked 7 days later and cloned cells were reestablished and restimulated in liquid cultures (*Fathman* and *Hengartner*, 1978). Colony formation by alloreactive T cells may be further improved by inclusion of macrophages in the bottom agar layer, and the probably inefficient stimulation by irradiated cells may be replaced by stimulation with mitogenic factors. At the present time, the development of techniques for cloning of alloreactive T-lymphocytes in semisolid media is in progress in several laboratories and a detailed description of the data would be premature.

4. Applications

a) Ig Expression in B Cell Clones

α) V Genes

All AFC that are members of an antigen- or mitogen-stimulated B cell clone are expected to secrete antibodies with identical specificities. This, however, is not easy to prove. Evidence was provided that monofocal antibodies produced by single spleen fragments were homogenous: electrophoretic mobility (*Klinman*, 1969; *Klinman*, 1971b) and isoelectric spectra (*Press* and *Klinman*, 1973a) were restricted as compared to normal serum antibodies, and monofocal antibodies bound hapten homogeneously as determined by a micromethod of equilibrium dialysis (*Klinman*, 1969; *Klinman*, 1971b); hapten-binding activity was completely regained when separated heavy and light chains of monofocal antibodies were recombined whereas combinations of separated heavy and light chains from heterologous antibodies showed at least a tenfold loss of antigen-binding capacity (*Klinman*, 1971a). In some cases, however, heterogenous antibodies were produced by apparently single B cell clones. *Macario* et al. (1972) found an increase in the antibody affinity of "monofocal" antibodies produced by rabbit lymph node fragments within several weeks of culture. *Luzzati* et al. (1973b) found rabbit lymphocyte clones isolated in microcultures producing antibodies that formed two bands on electrophoresis.

A more sensitive test for somatic mutations of V genes in stimulated B cells would be the analysis of antibodies produced by single AFC which are all the progeny of a single B cell. *Cunningham* and *Pilarski* (1974a, b) suggested that the plaque morphology could be used as a marker for the specificity of antibodies produced by single cells. Plaque assays were performed with mixtures of two different types of closely related indicator cells such as SRC and goat red cells, red cells from two different sheep, or red cells coupled with two different concentrations of a hapten (*Cunningham* and *Pilarski*, 1974b). Clear, partial, and sombrero plaques were thought to indicate AFC-secreting antibodies of different specificities. This interpretation was questioned (*Goldstein*, 1975) as well as supported (*De Lisi* and *Bell*, 1976) on theoretic grounds. Using this marker, variant cells were detected in clones of activated B cells. *Cunningham* and *Fordham* (1974) cultured single PFC isolated by micromanipulation for 2 days. Small clones developed from single cells, and 10 out of 93 clones had daughter cells producing different antibodies. In a similar experiment performed earlier by *Nossal* and *Lewis* (1971), no such variation was observed within the daughter cells of 13 isolated AFC.

Pilarski and *Cunningham* (1974) found more variants in B cell clones generated by LPS stimulation of cells in microcultures than was expected from chance overlap of two different clones. The clone size of cells secreting rare, highly cross-reactive antibodies was smaller than the average B cell clone size. This was interpreted to indicate that the small clones were derived from AFC initially producing other antibodies (*Pilarski* and *Cunningham*, 1974). Similar conclusions were drawn from studies on clones that were isolated in spleens of irradiated recipients (*Pilarski* and *Cunningham*, 1975).

β) C Genes

Investigation of the SRC response of peripheral blood lymphocytes from rabbits (*Luzzati* et al., 1973a) heterozygous at the b locus (b4 × b6) in limiting dilution cultures indicated that allelic exclusion of the Ig light chain allotypes is maintained throughout clonal proliferation as determined between the 12th and 21st days of culture (*Luzzati* et al., 1973b). The frequency distribution of b4 κ-chains, b6 κ-chains, and λ-chains was similar among anti-SRC antibody-secreting clones, surface Ig-positive peripheral blood lymphocytes and rabbit serum Ig.

The Ig class expression was studied mainly in mitogen-stimulated B cell clones. Previous culture conditions had allowed the in vitro development of IgM-secreting PFC after stimulation with LPS (*Melchers* and *Andersson*, 1973), but under improved conditions cells secreting other classes of Ig developed in LPS-stimulated cultures (*Kearny* and *Lawton*, 1975; *Melchers* et al., 1975; *Melchers* et al., 1976; *Andersson, J.* et al., 1976). With limiting numbers of LPS-reactive B cells, IgG antibody-secreting cells were shown to develop at day 7 in a small proportion of cultures containing IgM-secreting cells at day 5 (*Andersson, J.* et al., 1978). Analysis of surface Ig expression in cells from colonies grown in agar cultures failed to document a switch from IgM to IgG synthesis. Cells with surface IgG always also expressed IgM and were found early and late after initiation of the cultures (*Metcalf* et al., 1975b). *Wabl* et al. (1978) demonstrated unequivocally in cultures initiated with single LPS blasts that some cells producing only IgM could give rise to daughter cells producing IgG. These preliminary studies on cell differentiation in mitogen- or antigen-stimulated B cell clones have to be extended to the investigation of other cell surface markers, and the factors regulating the synthesis of different classes of Ig as well as the synthesis of secreted Ig versus cell surface-associated Ig have to be determined.

b) Frequency of Antigen-Reactive B Cells

Limiting dilution or spleen fragment cultures were used by many investigators to determine the frequency of B cells responsive under various culture conditions to SRC (*Quintans* and *Lefkovits*, 1973; 1974a, b, c; *Vann* and *Dotson*, 1974; *Halsall* and *Makinodan*, 1974; *Marbrook* and *Haskill*, 1974; *Hunter* and *Kettman*, 1974) or to various haptens coupled to T cell-dependent carriers (*Klinman* and *Press*, 1975b; *Hunter* and *Kettman*, 1974; *Sigal* et al., 1975; *Quintans* and *Cosenza*, 1976) or to haptens on T-independent carriers (*Hunter* and *Kettman*, 1974; *Cosenza* et al., 1975; *Quintans* and *Cosenza*, 1976; *Stocker*, 1976, 1977a).

All these studies were particularly useful for investigating various parameters of in vitro cultures influencing the cloning efficiency and the clone size of antigen-reactive B cells. Most studies were concerned with rather heterogenous PFC responses to red cells bearing many different antigenic determinants or to haptens to which a large spectrum of antibodies with different affinities for the hapten can be produced. The idiotype-restricted response of Balb/c spleen cells to phosphoryl choline (PC) was studied in limiting dilution cultures

(*Cosenza* et al., 1975; *Quintans* and *Cosenza*, 1976) or in the spleen fragment culture system (*Sigal* et al., 1975). Of the normal Balb/c spleen cells, $1–2 \times 10^{-5}$ responded to the heat-killed vaccine of pneumococci R36A (Pn) in microcultures with development of clones containing small numbers of anti-PC AFC, almost 90% of which expressed the idiotype characteristic for TEPC-15 myeloma PC-binding IgA molecules (*Cosenza* et al., 1975). This clearly indicated that the idiotype restriction found in Balb/c anti-PC responses is not due to clonal dominance. Less ($0.15–1.1 \times 10^{-5}$) but larger anti-PC AFC clones were induced with the T cell-dependent antigen PC-KLH (*Quintans* and *Cosenza*, 1976). When B cells were the limiting cell population, Pn and PC-KLH induced a higher fraction of responding wells than either antigen alone, suggesting that different anti-PC AFCP were stimulated by T-dependent and T-independent antigens (*Quintans* and *Cosenza*, 1976). *Sigal* et al. (1975) found also about 2 in 10^5 normal Balb/c spleen cells responsive to PC in spleen fragment cultures. The fragments were from hemocyanin-primed, irradiated Balb/c mice that were injected with normal Balb/c spleen cells, and the cultures were stimulated with PC coupled to hemocyanin via a tripeptide spacer (*Sigal* et al., 1975). Surprisingly, no anti-PC response was obtained in this system to Pn or to PC coupled directly to KLH (*Gearhart* et al., 1975a, b). Frequencies of antigen-specific B cells were also determined in mitogen-stimulated cultures. *Andersson, J.* et al. (1976, 1977a, b) found that 3%–30% of all B cells of various mouse strains could be stimulated with LPS or lipoprotein to proliferate and differentiate into Ig-secreting cells.

In limiting dilution cultures, high frequencies of mitogen-stimulated clones, secreting antibodies lysing SRC, or hapten-coupled SRC were found. With improved culture conditions (*Schreier* and *Nordin*, 1977), the same high frequencies of anti-SRC AFCP (1 per/2800 B cells) were found in SRC-stimulated cultures in the presence of activated T cells (*Schreier*, 1978, personal communication). Spleen cells from normal A/J mice contained 1 B cell in 2500 LPS-reactive cells which produced Ig expressing the A5A idiotype (*Eichmann* et al., 1977). Nearly half of the A5A idiotype-producing clones had binding affinity for the group A streptococcal carbohydrate (A-CHO). A tenfold increase in the frequency of A5A idiotype expressing LPS-reactive B cells was found in mice after immunization with antigen. Interestingly, a similar increase of cells producing A5A with and without binding affinity for A-CHO was observed in antigen-primed mice (*Eichmann* et al., 1977). This observation can be explained by a network theory of the immune system (*Jerne*, 1974).

c) Development of Antigen-Specific B Cells During Ontogeny

The specificity repertoire of lymphocytes is most likely acquired by somatic mutation of germ line V genes (*Tonegawa* et al., 1976). Knowledge of the specificity repertoire of lymphocytes may help to learn how diversity is generated. With rather limited knowledge of special features of the specificity repertoire of lymphocytes in adult animals (MHC-linked responsiveness and high frequency of alloreactive cells), *Jerne* (1971) postulated a mechanism for generation of diversity. We believe that *Jerne*'s model is correct for generation of diversity

in T-lymphocytes (*von Boehmer* et al., 1978b). From our knowledge about the B cell repertoire, we have no hint yet as to how Ig diversity may be generated. If *Jerne*'s model also applies to B cells, germ line V genes should be expressed early in B cell development. Thus, one approach would be to study the development of B cell specificities in ontogeny. This has been done by looking for immune responsiveness at various stages of development (*Silverstein* et al., 1963; *Sterzl* and *Silverstein*, 1967; *Sherwin* and *Rowlands*, 1975) or for antigen-binding lymphocytes (*Yung* et al., 1973; *Spear* et al., 1973; *Goidl* and *Siskind*, 1974; *D'Eustachio* and *Edelman*, 1975; *Lydyard* et al., 1976; *Cohen* et al., 1977).

The limitations of these studies are that lymphocytes binding a particular antigen and responding to a particular antigen are usually rather heterogenous. Moreover, the development of antigen responsiveness in ontogeny may depend not only on the development of a particular type of antigen-reactive lymphocyte but also on the development of auxiliary and interacting cells (*Argyris*, 1968; *Chiscon* and *Golub*, 1972; *Mosier* and *Johnson*, 1975). *Klinman* tried to circumvent these difficulties by testing for individual antigen-reactive B cells in neonatal, young, and adult mice using the spleen fragment culture system (*Press* and *Klinman*, 1973a, b; *Klinman* and *Press*, 1975a, b, c; *Klinman* et al., 1976a, b). Individual antigen-reactive B cells isolated in spleen fragments were stimulated in the presence of nonlimiting numbers of auxiliary cells and identified by the analyses of the isotopes, idiotypes, and isoelectric points of the products of their progeny (for review, see *Klinman* et al., 1976a, b). Results from such studies were interpreted to be consistent with the idea that generation of B cell diversity is based on antigen-independent preprogrammed mutations of germ line genes (for review, see *Klinman* et al., 1976a, b). Another approach was used by *Melchers* et al. (1976) who studied Ig synthesis and mitogen reactivity of fetal liver cells in suspension cultures. The earliest Ig-synthesizing cell was detected in the mouse at day 10 to day 12 of gestation (*Melchers* et al., 1975; *Phillips* and *Melchers*, 1976). Day 12 fetal liver cells developed in tissue culture into mature B-lymphocytes. LPS reactivity of B-lymphocytes, which is independent of auxiliary cells, was acquired around birth. Analysis of cultures containing limiting dilutions of fetal liver cells did not indicate any changes of the specificity repertoire of B cells grown in the presence of LPS (*Melchers* et al., 1976).

d) Frequency and Specificity of Alloreactive T Cells

Limiting dilution analysis of human alloreactive PBL in vitro was performed as early as 1969 by *Bach* et al. (1969). At least 1–10 cells in 2000 PBL were found to respond with proliferation to one particular type of allogeneic stimulator cell. Recently, several investigators determined the frequency of precursor cells of murine, cytotoxic T-lymphocytes in lymph node and spleen cell suspensions with specificity for allogeneic cells. *Teh* et al. (1977a) stimulated RNC nu/+ (H-2^k) or B6 nu/+ (H-2^b) lymph node of spleen cells in the presence of nu/nu accessory cells with H-$2^{k/d}$ or H-$2^{k/b}$ stimulator cells: 1 out of 800–900 lymph node cells and 1 out of 2550 spleen cells developed into a clone containing CTL with specificity for H-2^d target cells. The frequency of H-2^k CTLP reactive

to H-$2^{k/b}$ stimulator cells was 1 in 1350. H-2^k CTLP with specificity for H-2^d and H-2^b segregated independently of each other in limiting dilution cultures (*Teh* et al., 1977b). No cross-reactivities were observed, although H-2^d and H-2^b cells share some serologically defined H-2 determinants (*Klein*, 1975). Approximately 80% of lymph node cells are T cells, 7% of which represent the Ly 2,3 positive pool of cytotoxic T cells (*Cantor* and *Boyse*, 1975). This means that about 5%–6% of all lymph node cells are potential CTLP. The results obtained by *Teh* et al. (1977a, b, c) would indicate then that 2.1% of all potential CTLP from H-2^k mice were reactive to H-2^b cells and 1.3% to H-2^b. Very similar results were obtained by *Skinner* and *Marbrook* (1976), *Fischer-Lindahl* and *Wilson* (1977b), and *Watanabe* et al. (1977). Even higher frequencies of H-2^k lymph node cells with specificity for H-2^d and H-2^b were observed after stimulation with H-$2^{b/d}$ stimulator cells probably because back stimulation (*von Boehmer*, 1974; *von Boehmer* and *Sprent*, 1974) caused more low affinity cells to respond (*Teh* et al., 1977b).

In some experiments, cultures containing H-2^k responder cells, accessory cells, and H-$2^{d/b}$ stimulator cells were tested on day 7 (*Teh* et al., 1977c). Only half of the cultures were harvested for the assay, the remaining cells were incubated for a further 7 days, then restimulated with fresh H-$2^{d/b}$ stimulator cells, and tested for cytotoxic activity on H-2^d and H-2^b target cells 3 days after restimulation. The specificity of CTL in individual wells was identical in both the day-7 assay and the day-18 assay. However, only half of the cultures with cytolytic activity at day 7 were again cytolytic after restimulation (*Teh* et al., 1977c). Large clones containing about 1000 CTL were described by *Teh* et al. (1977a). The development of such clones from single precursor cells would require about ten cell divisions during an exponential growth for 5 days with a cell cycle time of 12 h, assuming that all members of the clone expressed cytolytic activity.

Stimulation of CTLP in microcultures with Con A was also dependent on accessory cells (*Teh* et al., 1977b; *Watanabe* et al., 1977). If tested in the presence of α-methylmannoside (to inhibit nonspecific lysis by Con A), 1 out of 1920 Con A-stimulated H-2^k lymph node cells developed clones with cytolytic activity for H-2^d target cells, and if tested in the presence of PHA (causing nonspecific lysis) (*Teh* et al., 1977b) 1 out of 40 Con A-stimulated H-2^k lymph node cells developed clones cytolytic for H-2^d target cells. This indicated that 2.5% lymph node cells could be induced to develop CTL clones, 2.1% of which were specific for H-2^d. Similar conclusions were drawn by *Bevan* et al. (1976) from studies on mitogen-stimulated bulk cultures.

Fischer-Lindahl and *Wilson* (1977b) found in limiting dilution cultures a few clones with specificity for third party target cells not cross-reactive with the specific stimulator cells probably due to some nonspecific activation and differentiation of CTLP in mixed lymphocyte cultures. *Skinner* and *Marbrook* (1976) found in limiting dilution cultures containing only responder cells a few clones with cytolytic activity for allogeneic target cells. Spontaneously arising clones from H-2^k cells lysed either H-2^d tumor cells or mitogen-induced H-2^d lymphoblasts but not both, while all clones induced with H-$2^{k/d}$ stimulator cells lysed both types of H-2^d target cells (*Ching* et al., 1977a). Spontaneously arising

CTL clones with specificity for syngeneic target cells were also observed (*Ching* et al., 1977a, b, c). Different target cells (tumor cells or lymphoblasts induced with various mitogens) were lysed by different clones (*Ching* et al., 1977c, d).

Preliminary experiments indicate that cloning of alloreactive T cells is also feasible in soft agar cultures after preselection of specific cells in long-term cultures (*Watanabe* et al., 1977; *Fathman* and *Hengartner*, 1978). Stimulatory supernatants from Con A-stimulated syngeneic, allogeneic, or xenogeneic mixed lymphocyte cultures are useful for cloning of alloreactive T cells.

e) Studies on Isolated T Helper Cells

Marrack (*Hunter*) et al. (1974) cultured 7×10^5–10×10^5 AT × BM spleen cells with 0–3×10^5 anti-T cell serum-treated spleen cells from mice injected 4 days before with SRC so that all cultures contained 10^6 cells in 1 ml medium. The cultures were stimulated with TNP-SRC, and anti-TNP IgM AFC were assayed 4 days later. AT × BM spleen cells alone gave no response, and the frequency of SRC-specific helper cells was determined from the fraction of nonresponding cultures containing limiting dilutions of helper cells. One million spleen cells contained 12.5 helper cells, one helper cell inducing an average of 28 anti-TNP PFC. In parallel experiments, graded numbers of spleen cells from mice immunized 6 days before intraperitoneally with KLH in CFA were incubated with 3×10^5 anti-T cell serum-treated DNP-BSA-primed spleen cells and TNP-KLH in 0.1 ml in tissue culture trays. Per 10^6 primed spleen cells, 2–30 helper cells were found, one helper cell inducing an average of 11.9 anti-TNP PFC (*Marrack* et al., 1974). The richest source of helper cells were spleen cells from immunized mice injected 6 days before with KLH in CFA (*Marrack* and *Kappler*, 1975). If limiting dilutions of these cells were added to B cell cultures (each culture containing at least one AFCP specific for SRC, BRC, and TNP), specific and nonspecific help by KLH-primed cells could be tested after stimulation with KLH and the appropriate antigens (*Marrack* and *Kappler*, 1975). KLH-primed cells producing nonspecific help for antibody responses to red cells segregated independently from KLH-primed cells producing specific help for anti-TNP AFC responses to TNP-KLH. Neither specific nor nonspecific helper activity was observed if normal unprimed B cells were used. Nonspecific help was only observed in anti-red cell AFC responses but not in AFC responses to soluble antigens (*Marrack* and *Kappler*, 1975).

In similar experiments using a microculture system (10 µl/cultures), SRC-activated T cells (present in the spleens of irradiated mice injected 7 days before with 10^8 syngeneic thymus cells and SRC) were shown to produce specific help for anti-SRC AFC responses (8×10^{-5} helper cells) and nonspecific help for anti-DRC AFC responses (at least 4×10^{-5} helper cells) of AT × BM spleen cells (*Waldmann* et al., 1975a). Long-term KLH-primed irradiated spleen cells also provided specific and nonspecific help for AFC responses of AT × BM spleen cells: 6.7×10^{-5} nonspecific helper cells for anti-SRC AFC responses and 1×10^{-5} specific helper cells for anti-TNP AFC responses to TNP-KLH (*Waldmann* et al., 1975a). Specific and nonspecific helper activity of nylon wool-

purified, irradiated, KLH-primed T cells were both shown to be long lived, ALS-sensitive T cells but to segregate independently in limiting dilution cultures (*Waldmann* et al., 1976b). IgM and IgG anti-TNP AFC were induced by different specific helper cells. The plaque size and the turbidity of plaques (using mixtures of indicator cells coupled with two different concentrations of TNP) in single clones was more homogeneous at limiting T cell input than at high T cell input (*Waldmann* et al., 1976b). In some experiments, TNP-primed B cell populations enriched in TNP-specific cells (unfortunately only twofold to threefold enrichment of anti-TNP AFCP was achieved using the gelatin separation technique) were used to increase the probability that one helper cell could interact with many different TNP-specific B cells. Again, clones presumably derived from only one AFCP were obtained with limiting numbers of T helper cells (*Waldmann*, 1977). These data were interpreted to indicate that one T helper cell was restricted to help only a limited number of B cells.

Nonspecific helper factors (NSF) were studied using also the Lefkovits microculture system. An NSF was obtained in the supernatants of short-term (48 h) cultures containing spleen cells from KLH-primed mice and KLH. The supernatant contained a factor that induced in the presence of SRC the majority of the available anti-SRC AFCP in nude spleen cells (as determined in the presence of irradiated allogeneic cells) or AT × BM spleen cells to proliferate and differentiate into AFC (*Lefkovits* et al., 1975). An NSF was also obtained in supernatants from spleen cultures (10^6 cells/ml) incubated for 24 h in the presence of 1 μg/ml Con A (*Waldmann* et al., 1976c). This Con A-induced NSF was produced by cells sensitive to ALS and not to adult thymectomy. The factor did not bind to anti-H-2 antibodies unlike the allogeneic effect factor (AEF) (*Armerding* and *Katz*, 1974) and the *Taussig* factor (*Taussig*, 1974; *Munro* et al., 1974; *Taussig* et al., 1975). In contrast to the effect of AEF (*Armerding* and *Katz*, 1974), there was no obvious strain specificity in the action of Con A-induced NSF on mouse B cell responses to SRC (*Waldmann* et al., 1976c). However, another restriction of Con A-induced NSF was suggested by a series of experiments in which NSF was produced in cultures containing limiting numbers of Balb/c spleen cells (10^4–10^5 per culture) *Lefkovits* and *Waldmann*, 1977). Supernatants were harvested from single cultures and several aliquots of each supernatant were transferred to cultures containing SRC and 1.5×10^5 nude Balb/c spleen cells. Only 1 in about 2×10^4 Con A-stimulated spleen cells generated helper activity for SRC, and the activity obtained from one well appeared to help only some anti-SRC AFCP but not others, suggesting that collaboration occurs only between matching T and B cell subsets (*Lefkovits* and *Waldmann*, 1977). The authors indicated that they reached the limits of their technical expertise in these experiments with limiting dilution cultures.

Prospects

In this review, we have described methods for separation, i.e., purification or elimination of specific lymphocytes on the basis of antigen binding or reaction to antigen. A number of applications have been discussed, the main limitations

being the lack of sufficient assay systems for specificity and function of lymphocytes.

Identification of V gene products requires a number of different markers. One particular combining site may be defined by its capacity to bind several determinants, i.e., several different antigens (fine specificity) as well as anti-idiotypic antibodies. A particular V gene may be linked to other genes encoding structures not directly involved in antigen binding such as allotypes or histocompatibility antigens. To provide tools for identification of specific cells and their receptors, it is important to further develop techniques for isolation, cloning, and/or hybridization of individual cells. The assessment of the function of individual cells is so far restricted to antibody-forming cells and killer cells. In the future, techniques will be required to identify not only all types of "executive lymphocytes" but also of antigen-reactive lymphocytes.

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Abbreviations

Antigens

Ag: antigen; BSA: bovine serum albumin; HSA: human serum albumin; GPA: guinea-pig albumin; OA: ovalbumin; FGG: fowl gamma globulin; HGG: human gamma globulin; KLH: keyhole limpet haemocyanin; MSH: spider crab haemocyanin; POL: polymerized flagellin; (T,G)-A-L: poly (Tyr,Glu)-poly DAla-poly Lys; TIGAL: iodinated(T,G)-A-L; TAA: tumor cell membrane extracts; BE: basic encephalitogen; SRC: sheep red cells; DRC: donkey red cells; BRC: burro red cells; CRC: chicken red cells; PRC: pig red cells.

Haptens

DNP: dinitrophenyl; TNP: trinitrophenyl; NIP: 3-iodo-4-hydroxy-5 nitro phenyl acetic acid; Fl (F): fluorescein; R: rhodamine; PC: phosphorylcholine; lac: lactoside; A-CHO: group A streptococcal carbohydrate.

Mitogens

LPS: lipopolysaccharide; PHA: phytohemagglutinin; Con A: concanavalin A; PWM: pokeweed mitogen.

Immunoglobulins

Ig: immunoglobulin; ab: antibody; M-Ig: membrane associated immunoglobulin; α-Ig: anti-immunoglobulin; α-Id: anti-idiotype.

Lymphocytes

ABC: antigen binding cells; RFC: rosette forming cells; AFC: antibody forming cells; PFC: plaque forming cells; AFCP: antibody forming cell precursors; B-MC: memory B-cells; CTL: cytotoxic T lymphocytes; CTLP: cytotoxic T lymphocyte precursors; BDL: blast derived lymphocytes; PEC: peritoneal exudate cells; PEL: peritoneal exudate lymphocytes; PBL: peripheral blood lymphocytes; TDL: thoracic duct lymphocytes; CRL: complement receptor bearing lymphocytes.

Immune Responses

CML: cell mediated lysis; MLC: mixed leukocyte culture; MLR: mixed leukocyte reaction; DTH: delayed type hypersensitivity; GvHR: graft versus host reaction; ADM: antibody dependent cell mediated cytolysis.

Various

ASLR: antigen specific lymphocyte recruitment; MHC: major histocompatibility complex; IU: immunocompetent units; ASU: antigen sensitive units; LU: lytic units; NSF: non specific helper factor; AEF: allogeneic effect factor; DMEM: Dulbecco's modified Eagle's medium; FCS: fetal calf serum; ^{3}H-TdR: tritiated thymidine; BUdR: 5-bromodeoxyuridine; IUdR: 5-iododeoxyuridine; ATxBM: adult thymectomized and bone marrow reconstituted; EDTA: ethylene diamine tetra acetic acid; MME: molar mercaptoethanol; cpm: cycles per minute.

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