

QUANTITATIVE FEATURES OF A SANDWICH  
RADIOIMMUNOLABELING TECHNIQUE FOR  
LYMPHOCYTE SURFACE RECEPTORS\*

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(Received for publication 21 September 1971)

The concept that lymphocytes recognize antigens by means of a surface receptor molecule of immunoglobulin (Ig) character has come to be widely accepted (1-3). Direct study of such receptors in the mouse by means of antiglobulin reagents labeled with either fluorescent (4, 5) or radioisotope (3, 6-8) markers has revealed a dense coating of surface immunoglobulins on nonthymus-derived or B lymphocytes<sup>1</sup> (5, 8), but the question of receptors on the surface of thymus-derived or T lymphocytes remains more controversial. On the one hand, immunofluorescent (5) and some radioimmunolabeling (6) techniques have failed to show immunoglobulin on the surface of T lymphocytes. On the other hand, treatment of T lymphocytes with specific rabbit anti-mouse globulin reagents has inhibited lymphocyte-antigen interactions such as rosette formation (9, 10) and radioactive antigen suicide (11). Moreover, in some (12-14) but not all (15) reports, *in vitro* binding of antiglobulin onto T lymphocytes has inhibited their capacity to mediate graft-vs.-host activity (12, 13), delayed hypersensitivity responses (12), or T cell dependent helper functions (14).

Recently, Bankhurst et al. (15) were successful in labeling up to 37% of some T lymphocytes with <sup>125</sup>I-labeled rabbit anti-mouse  $\kappa$ -chain IgG using exceptionally long radioautographic exposure periods. This prompted us to devise a "sandwich" radioimmunolabeling method, in which mouse lymphocytes were reacted first with a rabbit anti-mouse Ig reagent and then with a <sup>125</sup>I-labeled sheep anti-rabbit globulin (SARG). We hoped that extra sensitivity would be obtained which might allow labeling of an even higher proportion of T cells. Two further considerations favored a sandwich approach. First, it was a convenient way to compare directly the lymphocyte-binding power of a wide variety of different rabbit antisera without necessitating either fractionation or labeling of the rabbit sera. Secondly, we wished to study the metabolic

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\* Supported by grants from the National Health and Medical Research Council and the Australian Research Grants Committee, Canberra, Australia, and from the US Public Health Service (AI-O-3958 and AM-11234-04).

<sup>1</sup> *Abbreviations used in this paper:* BJP, Bence Jones protein; B lymphocyte, nonthymus-derived lymphocyte (see also text); BSS, balanced salt solution prepared as in Shortman et al.<sup>3</sup>; CFA, complete Freund's adjuvant; FCS, fetal calf serum; NRG,  $\gamma$ -globulin fraction of normal rabbit serum; NRS, normal rabbit serum; PAPS, polyaminopolystyrene; SARG,  $\gamma$ -globulin fraction of a potent sheep anti-rabbit Ig antiserum; SPF, specific pathogen-free; SRBC, sheep red blood cells; TDL, thoracic duct lymphocyte; T lymphocyte, thymus-derived lymphocyte (see also text).

characteristics of surface immunoglobulins. When lymphocytes were labeled at 0°C with antiglobulin-<sup>125</sup>I, washed, and then incubated at 37°C under tissue culture conditions, radioactivity soon appeared in the supernatant. The amount retained in the cells could have reflected either retention of label on the surface or pinocytosis of label. A sandwich assay, in which a second reagent "sees" the amount of first reagent left on the surface, seemed a convenient way of measuring the relative effects of these two processes.

In the present paper, we report the features of the sandwich assay, both in terms of bulk radioactivity counting of washed lymphocyte pellets and of radioautographic grain counts. It will be shown that the method readily discriminates between B and T lymphocytes, as the former label over 100 times more readily, and that nearly all T lymphocytes can be labeled by either anti- $\kappa$  or anti- $\mu$  reagents; but features demanding great caution in the interpretation of these results will emerge. A later paper<sup>2</sup> will show how the sandwich method has been used to establish a high rate of Ig receptor turnover in mouse B cells.

#### *Materials and Methods*

*Animals.*—Unless otherwise stated in the text, lymphocytes to be labeled were taken from unimmunized specific pathogen-free (SPF) C3H mice aged 10–12 wk. In a few experiments, mice were given 10<sup>9</sup> sheep erythrocytes intraperitoneally. Where lymphocytes from newborn mice were required, SPF C3H mice of both sexes were used. In some experiments, CBA and (CBA × C57BC)F<sub>1</sub> male mice (not SPF) aged 10–18 wk were used. Adult New Zealand rabbits, of either sex, were used for the preparation of anti-Ig sera.

*Antiglobulin Reagents.*—Rabbit antisera against mouse Ig chains were prepared using methods described in full elsewhere (16, 17). Briefly, rabbits were injected with 100–250  $\mu$ g of mouse immunoglobulin (prepared from normal mouse serum; the urine of mice carrying Bence Jones protein (BJP)—secreting plasmacytomas; a highly purified BJP or purified mouse myeloma proteins in complete Freund's adjuvant (CFA) in multiple sites. 3 wk later, the rabbits received 100  $\mu$ g of soluble protein intramuscularly, and over the next 1–3 yr the rabbits received periodic booster injections, usually in CFA, and were bled at various times. In general, the capacity of antisera to bind to T lymphocytes did not increase after the 1st 6 months of immunization. Sera prepared against mouse BJP usually displayed antibody activity against  $\kappa$ -chains only, and did not require absorption to render them specific. Sera prepared against either normal serum globulin fractions or mouse myeloma proteins were either used as bi- or polyvalent antiglobulin reagents, or were rendered specific against a particular immunoglobulin chain by suitable absorption procedures. These involved coupling appropriate myeloma proteins to the solid phase immunoabsorbent polyaminopolystyrene (PAPS) (16). After absorption, sera were tested for both the desired specific antiglobulin activity and residual contaminating activity against other chains by the sensitive radioimmunoprecipitation technique of Herzenberg and Warner (16). In the present paper, rabbit antisera prepared against BJP and not requiring absorption are identified simply by their number, e.g. R 14 or R 32; sera that are not monospecific are given a number followed by NS, e.g. R 19 NS; sera that have been rendered monospecific are followed by S, e.g. R 16 S; and sera that have had their specific antiglobulin antibody removed by solid phase absorption (i.e. are blocked) are followed by Bl, e.g. R 14-Bl or R 19 S-Bl. Normal rabbit serum is termed NRS and the 7S globulin fraction prepared therefrom, NRG.

<sup>2</sup> Wilson, J. D., G. J. V. Nossal, and H. Lewis. 1972. Metabolic characteristics of lymphocyte surface immunoglobulins. *Eur. J. Immunol.* In press.

The chief rabbit anti-mouse globulin sera used are described in Table I.

A hyperimmune sheep antiserum against rabbit immunoglobulin of extremely high activity (14 mg antibody/ml of serum) was kindly donated by Dr. Z. Ovary of New York University. A  $\gamma$ -globulin fraction of this (SARG) was prepared by starch block electrophoresis in Veronal buffer, pH 8.2. When direct labeling of lymphocytes with a rabbit antiglobulin was desired, a  $\gamma$ -globulin fraction of the rabbit serum was similarly prepared.

*Radioiodination of Gamma Globulins.*—The SARG or, on occasions, the  $\gamma$ -globulin fractions of various rabbit sera were labeled with carrier-free  $^{125}\text{I}$  (Radiochemical Center, Amersham, England) by the method of Greenwood et al. (18) using approximately 2 mg/ml of IgG, 100  $\mu\text{g}/\text{ml}$  of chloramine-T, usually 1 mCi of  $^{125}\text{I}/100 \mu\text{g}$  protein and reacting for 10 min at  $0^\circ\text{C}$ . Usually, the degree of labeling achieved was between 6 and 8  $\mu\text{Ci}/\mu\text{g}$  of  $\gamma$ -globulin, or ap-

TABLE I  
*Preparation and Properties of Rabbit Anti-Mouse Globulin Sera*

No. of serum	Immunogen* received by rabbit	Absorbing agent* coupled to PAPS	Antiglobulin activity of reagent
R 14	BJP from HPC-2	Nil	Anti- $\kappa$
R 14-B1	BJP from HPC-2	BJP from HPC-4	Nil detectable
R 19 NS	Normal NZB-IgM	Nil	Polyvalent, chiefly anti- $\kappa$ and anti- $\mu$
R 19 S	Normal NZB-IgM	HPC-1, HPC-5, HPC-32, RPC-5, and MPC-56	Anti- $\mu$
R 19 S-B1	Normal NZB-IgM	HPC-1, HPC-5, HPC-32, RPC-5, MPC-56, and MOPC-104	Nil detectable
R 18 S	HPC-4	MPC-76, MOPC-104, HPC-32, and GPC-5	Anti- $\alpha$
R 16 S	HPC-8	MPC-76, MOPC-104, HPC-1, and HPC-32	Anti- $\gamma_{2a}$ and anti- $\gamma_{2b}$
R 11 S	Normal mouse $\gamma$ -globulin	MOPC-104, MPC-86, HPC-13, and RPC-5	Anti- $\gamma_1$

\* Further details on the nature of the various myeloma proteins are given in references 16 and 17.

proximately 0.5–0.7 atoms of  $^{125}\text{I}/\text{molecule}$ . Labeled proteins were used as soon as possible after preparation, usually within 24 hr and always within 72 hr. Interestingly, it was found that, though prolonged storage at  $4^\circ\text{C}$  reduced the number of molecules of labeled reagent bound per lymphocyte, it did not appear to disturb the relative binding capacity of different reagents, but it was deemed prudent to avoid this complication of self-irradiation.

*Further Absorptions of Rabbit Antisera or SARG.*—In some experiments, SARG was absorbed with normal mouse  $\gamma$ -globulin on PAPS, or twice with washed mouse erythrocytes (20% packed cells, 30 min,  $0^\circ\text{C}$ ). It was found that these procedures did not affect the degree of binding of SARG- $^{125}\text{I}$  to mouse lymphocytes. Also, samples of rabbit antisera were absorbed twice with mouse erythrocytes, and/or one to eight times with washed mouse thymocytes (approximately  $5 \times 10^8$  cells/ml of serum, 30 min,  $0^\circ\text{C}$ ). The results of such absorptions will be described; the broad conclusion was that binding of rabbit antiglobulin to mouse lymphocytes was not due to contaminating anti-membrane antibodies.

*Preparation of Lymphocyte Suspensions.*—A key feature of our experimental design was to achieve interpretable labeling in cell suspensions as judged by bulk scintillation counting. It has

been a universal experience in this type of work that damaged cells and cell debris label non-specifically with many proteins. Accordingly, we used the method of Shortman et al.<sup>3</sup> to remove damaged cells and cell debris before labeling. This involved several steps, described in detail in their paper.<sup>3</sup> Briefly, large debris was first removed by allowing cell suspensions in 10% fetal calf serum (FCS)-balanced salt solution (BSS) (not yet centrifuged to a pellet) to stand for 5-15 min over 100% FCS, allowing large debris to settle well clear of the cells. The 10% serum layer containing cells and fine debris was then transferred onto another 100% FCS layer and centrifuged at 4°C in a swing-out head for 7 min at 400 g. The pellet represented relatively "debris-free" cells. Damaged cells were next removed on the basis of their having a higher density than healthy cells. This involved spinning the cells through bovine serum albumin solutions of density 1.0935 g/cm<sup>3</sup>, pH 5.1, at 3000 g for 10 min at 0°C. The pellet, containing damaged cells, was discarded. The supernatant, containing viable, debris-free cells, mixed with several times its volume of a 10% FCS-BSS isosmotic with mouse serum (308 milliosmols, equivalent to 0.16 M NaCl), buffered to pH 7.2 with isosmotic "Hepes" (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, Calbiochem, Los Angeles, Calif.), spun down at 400 g for 7 min, and adjusted to an appropriate cell concentration using the same 10% FCS-BSS.

*Preparation and Collection of Various Sorts of Thoracic Duct Lymphocytes.*—Thoracic duct cannulation was performed according to the method of Boak and Woodruff (19), and flow was assisted by continuous intravenous infusion. Thoracic duct lymphocytes (TDL) activated to react to allogeneic histocompatibility antigens ("T-TDL") were prepared according to the method of Sprent and Miller (20). Briefly, adult (CBA × C57BL)F<sub>1</sub> male mice were given 800 rads of whole body X-irradiation followed by administration of 10<sup>8</sup> CBA thymocytes, and 4 days later, TDL collection was started. TDL consisting chiefly of B lymphocytes (B-TDL) were harvested from the thoracic duct of adult CBA mice that had been thymectomized, irradiated with 800 rads, injected with 10<sup>7</sup> syngeneic bone marrow cells, and subjected to thoracic duct cannulation 4 wk later. Such B-TDL were found to contain only 10-17% of  $\theta$  positive cells (J. Sprent and A. Basten, unpublished).

*Direct and Sandwich Labeling Procedure.*—For direct labeling, lymphocyte suspensions in 10% FCS-BSS were mixed with 0.05-100  $\mu$ g of rabbit globulin-<sup>125</sup>I for 30 min at 0°C. Usually, experiments of both direct and sandwich type were done with samples of 0.2 ml volume, the cell concentration being 25 × 10<sup>6</sup>/ml for thymocytes and 10 × 10<sup>6</sup> ml for other lymphocytes, i.e., 5 × 10<sup>6</sup> and 2 × 10<sup>6</sup> lymphocytes total. After labeling, the cell suspension was layered over a discontinuous density gradient consisting of successive layers of 100% FCS; 75% FCS, 25% BSS; and 50% FCS, 50% BSS. This was centrifuged at 400 g for 7 min at 4°C, and the procedure was repeated. Cells were then resuspended in 0.2 ml and counted in a well-type scintillation counter.

For indirect or sandwich labeling, the procedure was as above, except that instead of <sup>125</sup>I-labeled rabbit globulin a dilution (from 1:10 to 1:100,000) of unfractionated rabbit serum was used as the first binding reagent. After 30 min reaction and two washes through FCS gradients, the cells were reacted for 30 min at 0°C with SARG-<sup>125</sup>I, at concentrations varying from 0.05  $\mu$ g/ml to 20  $\mu$ g/ml. Then, they were again washed twice through FCS gradients, resuspended, and counted.

In each experiment involving the sandwich technique, samples were included in which the first binding reagent was omitted (nil controls) or was an appropriate dilution of NRS (NRS controls).

<sup>3</sup> Shortman, K., N. Williams, and P. Adams. 1971. The separation of different cell classes from lymphoid organs. V. Simple procedures for the removal of cell debris, damaged cells and erythroid cells from lymphoid cell suspensions. *J. Immunolog. Meth.* Submitted for publication.

Preliminary experiments were run to determine the optimum period for binding at 0°C. Already at 5 min, some specific binding of antiglobulin to lymphocytes was noted, and this increased rapidly over the next 10 min, and slightly over the next 15 min. By 30 min, the system must have been near equilibrium as binding did not increase significantly over the next 90 min, so 30 min was chosen as the standard reaction time.

Extra washes beyond two were followed by some slight release of radioactivity into the supernatant, but the ratio of counts of samples reacted with antiglobulins to those reacted with NRS (specificity ratio) did not improve, so cycles of two washes were deemed sufficient. It has been shown previously that two washes through FCS gradients are more efficient in removing unbound radioactivity than five washes without gradients.

*Radioautographic Procedure.*—After scintillation counting, cell suspensions were layered over and spun through 100% FCS. The supernatant was removed, and a few microliters of FCS, sufficient to make a suspension of about 10% white cells (v/v) was added. After vigorous mixing, air-dried smears on gelatin-coated slides were prepared and fixed in 90% methanol. Three to five replicate smears of each cell suspension were prepared. These were dipped in "Kodak" NTB/2 liquid radioautographic emulsion (Eastman Kodak Co., Rochester, N. Y.) and exposed for periods varying from 2 hr to 70 days (usually 3, 10, and 60 days). After development and staining with Giemsa stain, smears were examined for per cent labeled cells (where 300–1000 cells were scored) and for grain count frequency distribution (where 100 cells were scored). If, for any reason, it was desired to obtain more information on rare, large cells, the edges of smears were appropriately scanned.

In many cases, wide variation from cell to cell in grain counts was observed, and it was not easy to decide whether a cell was "labeled" or "unlabeled." An arbitrary cut-off point of 10 grains/cell was chosen as the threshold of labeling.

As will emerge under Results, T lymphocytes labeled much more lightly than B lymphocytes with all antiglobulins tested. To allow direct comparison of grain counts for T and B cells, it was necessary to use widely varying exposure periods. Observed grain counts were accordingly adjusted by a factor appropriate for the actual exposure period and the half-life of the isotope (60 days). No allowance was made for latent image fading, which we have not found to be a significant factor in this work.

## RESULTS

*Comparison of Direct and Sandwich Techniques Using Spleen or Thymus Lymphocytes.*—We wished first to test the postulate that the sandwich method had greater sensitivity than the direct labeling method, and to establish suitable boundary conditions for direct and sandwich labeling. In Fig. 1, the relative binding to spleen cells of  $\gamma\text{G-}^{125}\text{I}$  from R 14 (anti- $\kappa$ ) and of NRG- $^{125}\text{I}$  is compared over a wide range of concentrations of the labeling reagents. It can be seen that the degree of nonspecific binding of NRG is strictly proportional to concentration over the whole range tested, whereas with the specific anti- $\kappa$  reagent, there is proportionality up to a concentration of 30  $\mu\text{g}$  R 14- $\gamma\text{G-}^{125}\text{I}/\text{ml}$ , followed by a rate of increase only slightly greater than that seen with NRG- $^{125}\text{I}$ . This suggests some degree of saturation of receptors round 30  $\mu\text{g}/\text{ml}$ . At this concentration, calculations based on the substitution rate of the labeling reagent and the efficiency of the scintillation counter suggested a mean number of about 30,000 molecules of anti- $\kappa$  globulin bound per spleen lymphocyte.

When the same two samples of iodinated reagents were tested on thymus lymphocytes, the surprising finding was noted that R 14-IgG- $^{125}\text{I}$  did not bind

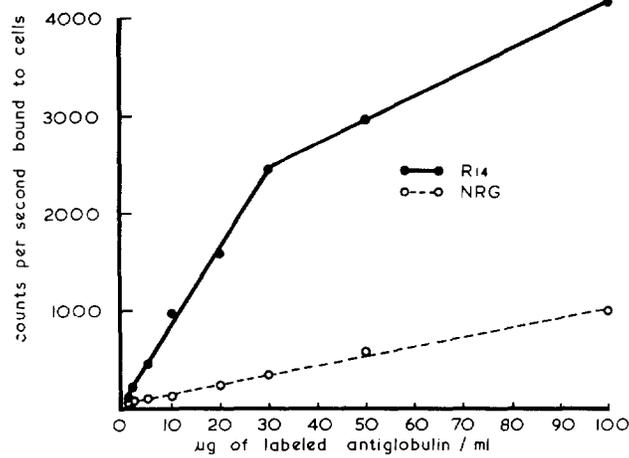


FIG. 1. Bulk scintillation counts of mouse spleen cell suspensions after labeling by the direct method with either an anti- $\kappa$ , IgG- $^{125}\text{I}$ , or NRG- $^{125}\text{I}$  at various concentrations.

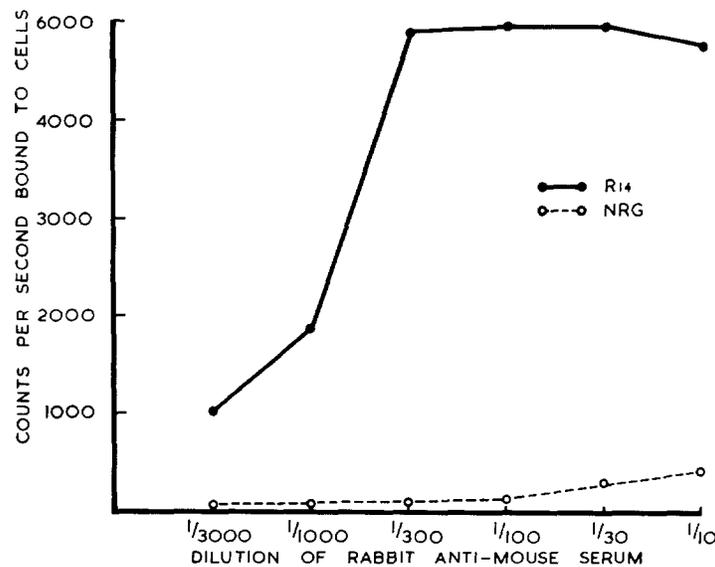


FIG. 2. Bulk scintillation counts of mouse spleen cell suspensions after labeling by the sandwich method. The first reagent was either an anti- $\kappa$  serum or NRS, the second reagent, SARG- $^{125}\text{I}$  at  $5\ \mu\text{g}/\text{ml}$ .

to a greater extent than NRG- $^{125}\text{I}$  at any concentration tested, as judged by bulk radioactivity counting. This becomes a little easier to understand when it is realized that, under the conditions used, only 0.02% of the radioactivity originally present in the reaction mixture remained associated with the cell

pellet in the case of thymus cells. Clearly, nonspecific carry-over of radioactivity becomes a substantial factor in experiments of such design.

In Fig. 2, the results of an experiment using the sandwich method on spleen cells are shown. The first reagent was either R 14 or NRS at concentrations varying from 1:3000 to 1:10, and the second reagent, SARG-<sup>125</sup>I at 5  $\mu$ g/ml. Two points appear immediately. First, the specificity ratio at all concentrations, as judged by bulk radioactivity counting, is greater than with the direct method.

TABLE II  
*Labeling of Mouse Spleen or Thymus Lymphocytes by Antiglobulin Reagents under Various Conditions*

Organ	Labeling conditions	Counts/sec per 10 <sup>6</sup> cells	Specificity ratio*	Lymphocytes labeled† %	Mean grain count‡
Spleen	1:10,000 R 19 NS; 0.2 $\mu$ g/ml SARG- <sup>125</sup> I	36.2	20.7	49	38.6
	1:10,000 NRS; 0.2 $\mu$ g/ml SARG- <sup>125</sup> I	1.75		1	<1
	0.2 $\mu$ g/ml R 19 NS- <sup>125</sup> I	33.5	16.6	50	33.9
	0.2 $\mu$ g/ml NRG- <sup>125</sup> I	2.02		0	<1
	1:500 R 19 NS; 2 $\mu$ g/ml SARG- <sup>125</sup> I	1248		100	1402
Thymus	1:500 R 19 NS; 2 $\mu$ g/ml SARG- <sup>125</sup> I	36.1	7.1	79¶	21.5
	1:500 NRS; 2 $\mu$ g/ml SARG- <sup>125</sup> I	5.1		3	<1
	20 $\mu$ g/ml R 19 NS- <sup>125</sup> I	33.6	3.8	59	15.3
	20 $\mu$ g/ml NRG- <sup>125</sup> I	8.8		1	<1
	1:10,000 NRS; 0.2 $\mu$ g/ml SARG- <sup>125</sup> I	1.0		0.6	<1

\* Ratio of number of scintillation counts/sec in cell pellet after labeling with antiglobulin reagent vs. after normal rabbit serum control.

† A cell was considered labeled if covered or surrounded by 10 or more grains.

‡ The mean refers to all cells whether labeled or unlabeled, in order to allow comparison with liquid scintillation counts. Actual grain counts have been standardized for equivalent exposure periods (see Materials and Methods), in this case 5 days.

|| A small adjustment was made to the actual counts to allow for slightly lower <sup>125</sup>I substitution of NRG vs. R 19 NS. The degree of iodination of R 19 NS and SARG was identical.

¶ The per cent of labeled thymocytes depends on exposure time; see text.

Secondly, there is a clear plateauing of binding of the first reagent (as read by subsequent binding of the second reagent) at concentrations of R 14 above 1:300. If we presume that this hyperimmune rabbit had 10 mg/ml of  $\gamma$ G per milliliter of serum, this plateau would be at 33  $\mu$ g/ml of R 14- $\gamma$ G, thus fitting in well with the findings suggesting saturation with the direct technique.

Experiments of similar design were undertaken with serum R 19 NS, a very strong polyvalent rabbit anti-mouse globulin reagent. Results of a typical experiment are given in Table II. From the bulk counts, it emerges that significant signal-to-noise ratios for thymus cells can be obtained with the direct method,

but the sandwich method still carries an advantage in this regard. The bulk counts also show that with the direct method, it took 100 times more R 19 NS- $\gamma$ G- $^{125}$ I in the reaction mixture to bind the same number of counts to  $10^6$  thymus cells as to  $10^6$  spleen cells. When identical conditions of indirect binding were used, spleen cells bound about 40 times the number of counts than did thymus cells. This suggests that, on the average, spleen cells bind antiglobulin much more extensively than do thymus cells.

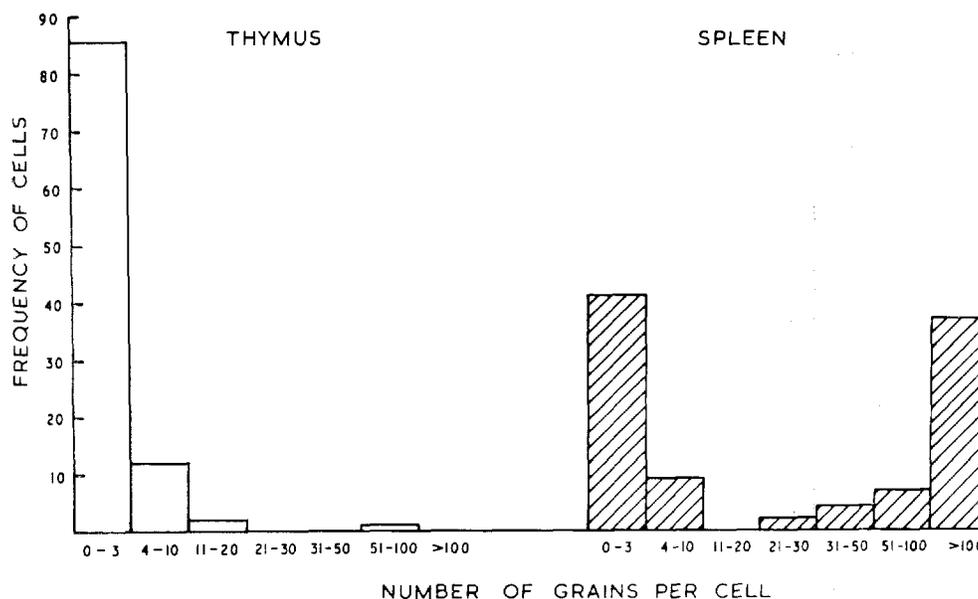


FIG. 3. Grain count frequency histogram of thymus or spleen lymphocytes labeled by the sandwich method. Labeling reagents: R 14 at 1:300; SARG- $^{125}$ I at 5  $\mu$ g/ml; exposure of radioautographs, 3 days.

Radioautographic analysis revealed a number of further points. First, as has been repeatedly reported, spleen cells could be divided into two populations, namely easily labeled and labeled only with much higher concentrations of antiglobulin (see also Fig. 3 below). Secondly, all spleen lymphocytes and most thymus lymphocytes could be labeled by R 14 under conditions where only few cells were labeled with NRS. Thirdly, the direct method gives much better specificity ratios for thymus cells using radioautography than using bulk counts. This is presumably because contaminating radioactivity, not firmly bound to cells, is either washed away during fixing and washing of smears, or scattered randomly between the cells on the smear. Fourthly, the grain counts show an even greater difference between thymus and spleen cells in capacity to bind R 19 NS than do the bulk counts. For example, the factors of 100 and 40

mentioned above become 220 and 70, respectively. Bearing in mind that virtually all the grains over spleen cells come from about 50% of the population, it is clear that these cells in spleen bind from 140 to 440 times more R 19 NS than the majority of cells in thymus, as judged by radioautography.

From these results it might be expected that it would be difficult to find labeling conditions such that, on one and the same radioautograph, binding to heavily labeled and lightly labeled cells could be directly compared. Grains become confluent and uncountable around 100/cell. However, in Fig. 3 we present an attempt to achieve this goal. Thymus or spleen cells were labeled with R 14 at 1:300 and SARG-<sup>125</sup>I at 5  $\mu$ g/ml. After 3 days' exposure, thymus cells were just beginning to display some slight labeling. Only an occasional cell exhibited >10 grains. Spleen cells fell into two groups. About half of them behaved like thymus cells, being unlabeled or very lightly labeled. Half of them were moderately to heavily labeled, there being 37% of cells showing >100 grains/cell. In many of these cases, the cell was covered by confluent grains and a halo of grains spread out around the cell, suggesting that the grain count exceeded 100 by a considerable margin. Such "high density surface Ig" lymphocytes could be labeled under conditions (e.g. 1:10,000 rabbit antiglobulin; 0.2  $\mu$ g/ml SARG-<sup>125</sup>I; 5 days' exposure) that left thymus lymphocytes totally unlabeled.

We felt the capacity of antiglobulin sera to bind to thymus cells to be encouraging, and thus investigated a large number of anti- $\kappa$  sera, including five bleeds of R 14 taken at intervals during the immunization schedule. The results are given in Table III. A number of important methodological points emerged. First, though all the anti- $\kappa$  sera bound to spleen cells, only some bound to thymus cells to a significantly greater extent than did NRS, i.e., with a specificity ratio >2. The spleen/thymus binding ratios fluctuated between 16 and 52. Secondly, relatively early bleeds of R 14 were highly effective, and though relevant results are not shown on the table, this was equally true of many other sera used in the present work. Thirdly, NRS binding is considerably higher with spleen than with thymus. This was a consistent finding, and was true both for binding of the first reagent, NRS, and for binding of SARG-<sup>125</sup>I itself in the absence of any first reagent. Several factors contribute to this, including the presence in spleen cell suspensions of many platelets and some macrophages and polymorphs (that bind protein by processes that appear to be nonimmunological), and a slight residuum of dead cells and debris in spleen cell suspensions.

*Immunological Nature of Binding of Rabbit Sera to Mouse Thymocytes.*—It next became important to determine whether the binding of rabbit sera to mouse thymocytes was due to their content of anti-mouse globulins. Table IV lists some relevant results. About 140 different samples of rabbit sera, of which 38 are shown, were tested for their ability to bind to thymocytes as judged by the sandwich assay and bulk scintillation counting. Antisera from rabbits which

had been immunized with a variety of antigens in CFA, including many human myeloma proteins, did not bind to thymocytes to a significantly greater extent than did NRS. Anti-lymphocyte sera gave a much greater degree of binding than even the strongest antiglobulin sera. This shows how careful one must be to exclude the presence of anti-membrane antibodies in work of this kind. That point is further brought out by the results obtained with antisera against chicken bursal extract or chicken thymus extract. These were the only other sera lacking detectable activity against mouse immunoglobulins which bound to thymocytes, and we interpret this to suggest an immunological cross-reaction

TABLE III  
*Capacity of Various Anti- $\kappa$  Chain Sera to Label Mouse Spleen or Thymus Lymphocytes\**

Serum	Spleen		Thymus		Spleen: thymus ratio
	Counts/sec per 10 <sup>6</sup> cells	Specificity ratio	Counts/sec per 10 <sup>6</sup> cells	Specificity ratio	
R 14-1	576	6.1	16	1.0	36
R 14-2	1784	19	63	3.9	28
R 14-3	2024	22	61	3.8	33
R 14-4	1560	17	47	2.9	33
R 14-5	1305	14	25	1.6	52
R 32	791	8.4	46	2.9	17
R 36	617	6.6	30	1.9	21
R 37	934	9.9	57	3.6	16
R 49	449	4.8	26	1.6	17
R 50	346	3.7	35	2.2	16
R 51	418	4.4	26	1.6	16
NRS	94	—	16	—	5.9

\* Labeling conditions: 1:300 serum; 5  $\mu$ g/ml SARG-<sup>125</sup>I.

between chicken lymphocyte membranes and mouse lymphocyte membranes, not shared by the rabbit. Of the rabbit anti-mouse globulin reagents, polyvalent antiglobulins tended to give better binding than anti- $\kappa$  sera, and an anti- $\lambda$  serum did not bind. There was not always close correlation between the anti- $\kappa$  strength of a serum and its thymus-binding capacity. For example, with several sera the thymus-binding strength fell but the anti- $\kappa$  strength rose with prolonged immunization.

Further and more persuasive evidence of the immunological specificity of binding of antiglobulins to thymocytes is given by blocking experiments in which the antiglobulin activity of a serum was absorbed out by a specific globulin on PAPS. Results of a typical experiment are given in Table V. When the polyvalent antiglobulin R 19 NS was rendered monospecific for  $\mu$ -chains, the ability to bind to thymus or spleen cells fell by an approximately equal percentage. When the residual specific anti- $\mu$  activity was removed, a further three- to fourfold drop in binding ensued, though some slight ability to bind to

TABLE IV  
*Specificity of Binding of Rabbit Sera to Mouse Thymocytes As Judged by a Sandwich Radioimmunoassay*

Immunogen*	Relative‡ binding§ (NRS = 100)	Immunogen	Relative binding (NRS = 100)
<i>Salmonella adelaide</i> O antigen	95	Mouse thymocytes-1	35,600
<i>Salmonella adelaide</i> polymerized flagellin-1	97	Mouse thymocytes-2	38,200
		Normal mouse globulin	1180
<i>Salmonella adelaide</i> polymerized flagellin-2	109	Normal NZB mouse IgM	1110
		Normal NZB mouse IgM	695
Mouse muramidase (urine concentrate from monocytic leukemia)	137	Normal mouse $\gamma$ -globulin	900
		Mouse BJP protein (urine concentrate) from HPC-18	855
DNP-human serum albumin	154	Mouse BJP protein (urine concentrate) from HPC-10	336
DNP-chicken gamma globulin	130	Mouse BJP protein (urine concentrate) from GPC-7	545
Human $\kappa_1$ BJP	125	Mouse BJP protein (urine concentrate) from HPC-11	190
Human $\kappa_2$ BJP	61	Mouse BJP protein (highly purified) from HPC-2	384
Human myeloma $\lambda G_1$	121	Mouse BJP protein (highly purified) from HPC-14	305
Human myeloma $\kappa G_1$	132	Mouse Fab of IgG	242
Human myeloma $\lambda G_2$	91	Mouse myeloma globulin MPC-8 (IgG <sub>2b</sub> )	193
Human myeloma $\kappa G_3$	60	Mouse myeloma globulin HPC-3 (IgG <sub>2a</sub> )	154
Human myeloma $\kappa$ IgM	98	Mouse myeloma globulin MPC-4 (IgA)	390
Human myeloma $\lambda$ IgA	87	Mouse myeloma globulin MOPC-104 (IgM)	223
Human myeloma $\kappa$ IgA	108	$\lambda$ -chains from MOPC-104	78
Chicken H chains of IgG	149		
Chicken L chains of IgG	56		
Chicken bursal extract	605		
Chicken thymus extract	332		

\* Rabbits were immunized with the various antigens in CFA and regularly boosted. See Materials and Methods.

‡ The results are pooled from several separate experiments. In each experiment, one to three normal rabbit sera were included at equivalent dilution as controls. Variance amongst individual NRS was small. The mean NRS count rate was determined, and all other count rates expressed as a percentage of it, to allow comparison between experiments.

§ The binding assay involved reacting mouse thymocytes with rabbit antisera at dilutions from 1:200 to 1:500; and then with SARG-<sup>125</sup>I at 5  $\mu$ g/ml.

|| BJP = Bence Jones protein.

both thymus and spleen cells was retained. When the anti- $\kappa$  reagent R 14 was absorbed with a  $\kappa$ -BJP (different from the immunogen) on PAPS, again a significant reduction, greater for spleen than for thymus, in binding capacity was achieved. It is of interest to note that, though no trace of antiglobulin activity

could be detected in the blocked sera by the radioimmunoprecipitation method, blocking failed to remove totally the lymphocyte-binding capacity.

Further control experiments were performed to exclude a role of anti-membrane antibodies (other than anti-immunoglobulin antibodies) in the thymus labeling phenomenon. Polyvalent antiglobulin sera or anti- $\kappa$  sera were absorbed twice with mouse erythrocytes or one to eight times with mouse thymocytes. Such treatments lowered the binding of both specific sera and of NRS to thymocytes, slightly and to a variable extent, and did not improve the signal-to-noise ratio. In a typical experiment, five successive absorptions with

TABLE V  
*Specificity of Binding of Rabbit Antiglobulin Reagents to Mouse Spleen or Thymus Lymphocytes As Tested by Blocking Reactions\**

Rabbit reagent	Thymus cells‡		Spleen cells§	
	Counts/sec per 10 <sup>6</sup> cells	Mean grain count	Counts/sec per 10 <sup>6</sup> cells	Mean grain count
R 19 NS	100	100	100	100
R 19 S	45	35	30	31
R 19 S-B1	15	15	9	8
NRS	6	1.5¶	4	2.9¶
R 14	100	100	100	100
R 14-B1	40	36	24	19
NRS	11	7¶	6	5¶

\* For convenience of comparison, results have been normalized so that the two unabsorbed sera R 19 and R 14, equal 100.

‡ Conditions of labeling: 1:200 rabbit serum; 5  $\mu$ g/ml SARG-<sup>125</sup>I.

§ Conditions of labeling: 1:10,000 rabbit serum; 1  $\mu$ g/ml SARG-<sup>125</sup>I.

|| The nonspecific binding of NRS to lymphocytes was not reduced by absorption with mouse immunoglobulins on PAPS.

¶ The apparently positive labeling in these controls arises from the normalization procedure. In fact, the mean grain count in all cases was around 1 grain/cell.

thymus cells halved both values. At the same time the antiglobulin titer was lowered to an approximately equivalent extent. The results were what would be expected if thymus cells had a small amount of immunoglobulin on their surface, and quite different from those expected on the basis of a natural or acquired anti-mouse lymphocyte membrane antibody.

*Incidence of "High Surface Density Ig" Lymphocytes in Various Organs.*—Next, we wanted to use the sandwich assay to confirm the results of others (5-7) with the direct assay on the relative frequency of high surface density Ig lymphocytes in various organs. Pooled results of a large series of experiments are given in Table VI. The results strongly suggest that the "easily labeled" or "high Ig density" lymphocytes are indeed B cells. Three known sources of T lymphocytes, namely thymus, H-2 activated, thymus-derived TDL (T-TDL),

and sheep red blood cell (SRBC)-activated thymus-derived spleen cells, yielded few or no easily labeled cells. A source of cells rich in B cells, and known to contain only 10-17%  $\theta$  positive cells, namely, TDL from adult, thymectomized, irradiated, bone marrow-protected mice (B-TDL), yielded 72.3% high Ig density cells, and about 10% of the remaining cells were plasma cells, many of

TABLE VI  
*Incidence of Lymphocytes Capable of Binding Anti- $\kappa$  Sera at Low Dilution\* (High Surface Density Ig Lymphocytes)*

Source of lymphocytes	No. of experiments	Mean per cent of labeled lymphocytes
Adult SPF spleen	9	41.7
Adult SPF thymus	3	0.2
Adult SPF lymph node	2	21.5
Adult SPF Peyer's patch lymphocytes	1	53
Adult "conventional" spleen	1	46
Adult 4-5-day immunized spleen‡	2	52
Newborn SPF spleen	1	4
7-day-old SPF spleen	1	10
Newborn SPF thymus	1	0
7-day-old SPF thymus	1	0
"Conventional" thoracic duct lymphocytes	2	15.1
"H-2 activated", thymus derived, thoracic duct lymphocytes ("T-TDL")§	2	0.5
SRBC-activated T cells, harvested from spleen	1	1
Thoracic duct lymphocytes from AT $\times$ XBM mice ("B-TDL")¶	1	72.3

\* Conditions of labeling: between 1:10,000 and 1:1000 anti- $\kappa$  serum; 0.2 to 1  $\mu$ g/ml SARG-<sup>125</sup>I; exposure period of radioautographs 1-4 days. The conditions were designed to label B lymphocytes but not T lymphocytes. See text.

‡ Immunization was a single intraperitoneal injection of  $10^9$  SRBC.

§ CBA  $\times$  C57BC mice were given 800 rads of whole body X-irradiation, and  $10^8$  thymus cells from an adult CBA mouse intravenously. 4-5 days later, lymphocytes were harvested from the thoracic duct.

|| CBA mice were given 800 rads of whole body X-irradiation,  $10^8$  syngeneic thymus cells,  $10^9$  SRBC, and 6-7 days later were killed and the spleen harvested.

¶ Adult CBA mice were thymectomized, irradiated, injected with  $10^7$  syngeneic bone marrow cells, and 4 wk later, lymphocytes were collected from the thoracic duct ("B-TDL").

which are not rich in receptors by the present technique (4, 21). Lymph nodes showed fewer, but Peyer's patches more, high Ig density cells than did spleen, and immunization with  $10^9$  sheep erythrocytes may have slightly raised the proportion of labeled cells. Some high Ig density cells of typical small-to-medium lymphocyte morphology were present in the spleen already at birth. For convenience, we shall use the terms easily labeled lymphocyte, high density surface Ig lymphocytes, and B cells interchangeably in the rest of this paper.

*Labeling of Spleen B Cells with Various Monospecific Anti-Heavy Chain Sera.*—An “all-or-none” classification of lymphocytes into easily labeled (or B) and difficult to label (or T) categories proved easy with polyvalent antiglobulin, anti- $\kappa$ , or anti- $\mu$  reagents, but such was not the case when anti- $\gamma_1$ , anti- $\gamma_2$ , or anti- $\alpha$  sera were used at equivalent concentrations. A typical experiment is shown in Table VII. Here it can be seen that, with 1:1000 rabbit antiglobulin followed by 1  $\mu\text{g}/\text{ml}$  SARG- $^{125}\text{I}$ , and an exposure period of 4 days, anti- $\kappa$  and anti- $\mu$  reagents each bind to about one-third of the spleen lymphocytes, and grain counts over most labeled cells were heavy. With the anti- $\gamma_2$  and anti- $\alpha$  reagents, there were fewer labeled cells, but considerable numbers of cells

TABLE VII  
*Labeling of Spleen Lymphocytes Using Reaction Conditions Favorable for B Cell Labeling\* and Various Exposure Times*

No. of serum	Activity of serum	Counts/sec per $10^6$ spleen cells	Per cent labeled lymphocytes		
			4 day exp.	11 day exp.	70 day exp.
Nil†	Nil	60	0	0.7	4.7
R 350	NRS	143	0	2.3	8.3
R 14	Anti- $\kappa$	842	35.5	37	66.7
R 19 S	Anti- $\mu$	587	32.7	33.4	57.3
R 11 S	Anti- $\gamma_1$	142	1.3	2.0	18.3
R 16 S	Anti- $\gamma_2$	437	5.7	22.7	62.7
R 18 S	Anti- $\alpha$	391	10.5	28	48.7

\* Cells were reacted with 1:1000 rabbit anti-mouse globulin serum and 1  $\mu\text{g}/\text{ml}$  SARG- $^{125}\text{I}$ . Under such conditions, exposure periods of 1–4 days are adequate for B cells. See text.

† Control where no rabbit serum but only SARG- $^{125}\text{I}$  was used.

displayed grain counts just under the threshold value of 10 grains. As the exposure period was lengthened to 11 days, the proportion of cells labeling with anti- $\kappa$  and anti- $\mu$  did not change significantly, but the proportion labeling with anti- $\gamma_2$  and anti- $\alpha$  sera rose sharply. The table makes the further point that labeling reactions of the present type must always be calibrated against relevant controls; with a 70 day exposure period, nonspecific binding of SARG- $^{125}\text{I}$ , though at light grain count, began to be a substantial nuisance.

At face value, the above results suggested that mouse B cells might display more than one heavy chain class at their surface. As <5% of mouse light chains are of  $\lambda$  type (22), one can as a first approximation regard the lymphocytes labeling easily with anti- $\kappa$  as the sum total of B lymphocytes, and even with the shortest exposure times, the total of cells labeling with any anti-heavy chain serum (50.2%) exceeds the proportion labeling with anti- $\kappa$  (35.5%). In Table VIII, we present pooled data of several experiments which reinforce this point. The results show that, under the gentle B cell conditions, no T cells labeled with any of the anti-heavy chain reagents. Again regarding R 14

labeling as indicating B cell status, there is clear evidence that not all B cells displayed only one heavy chain class; the sum total of labeling percentages with anti- $\mu$  + anti- $\alpha$  + anti- $\gamma_2$  + anti- $\gamma_1$  considerably exceeded the percentage labeled with anti- $\kappa$ .

When this question was analyzed taking into account the diameter of labeled cells, this "heavy chain superaddition" phenomenon became more marked. Relevant results are given in Table IX. This shows that the smallest cells (<6.5  $\mu$  diameter, some of which might possibly have represented erythrocyte precursors) rarely labeled with any antiglobulin, but that cells over 10  $\mu$  diameter labeled more frequently than the total population with several of the anti-heavy

TABLE VIII

*Labeling of Various Types of Lymphocytes with Specific Anti-Heavy Chain Sera under Reaction Conditions Favorable for B Cell Labeling*

No. of serum	Activity of serum	Per cent lymphocytes labeled			
		"Conventional" spleen	"Conventional" TDL	B-TDL	T-TDL
Nil	Nil	0	0	0	0
R 350	NRS	0	0	0	0
R 14	Anti- $\kappa$	46	15.2	72.3	0
R 19 S	Anti- $\mu$	46	9.8	57	0
R 18 S	Anti- $\alpha$	28.5	11.5	46.3	1
R 16 S	Anti- $\gamma_2$	18.5	} 5.8*	11.3	} 0*
R 11 S	Anti- $\gamma_1$	2.5		N.D.	

\* Because of shortage of cells, reagents R 16 S and R 11 S were pooled.

chain reagents. Again taking the results at face value, this could imply that most large cells displaying  $\kappa$ -chains and  $\mu$ -chains on their surface also simultaneously displayed  $\gamma_2$  and/or  $\alpha$ -chains, a claim made previously by others (23, 7, 24). This issue will be taken up in the discussion.

*Labeling of Thymus Cells with Various Anti-Heavy Chain Reagents.*—When thymus cells or T-TDL were labeled under the gentle B cell conditions (i.e. 1:10,000–1:1000 rabbit antiglobulin followed by 0.2 to 1  $\mu\text{g}/\text{ml}$  SARG- $^{125}\text{I}$ , and exposure periods of 1–5 days), virtually no labeling was encountered. However, it was possible to raise the concentrations of the two reactants to "T cell labeling conditions" and to achieve labeling with antiglobulin reagents but not with NRS controls. T cell conditions involved 1:200–1:300 rabbit antisera as the first binding reagent and 5  $\mu\text{g}/\text{ml}$  SARG- $^{125}\text{I}$ . Results of a typical experiment are given in Table X. This shows that with 3 days' exposure, over half the thymus cells labeled with anti- $\kappa$  serum; nearly all labeled with either polyvalent antiglobulin or, more lightly, with an anti- $\mu$  serum; only 1% labeled with NRS or SARG- $^{125}\text{I}$  alone; and substantial partial blocking of labeling could be achieved by absorbing out the specific anti- $\kappa$  or anti- $\mu$  activity. As the exposure

period was lengthened, three things were observed. First, up to 4.3% of cells in control smears showed label, although of light grain count. Secondly, the

TABLE IX  
*Size Range Distribution of Spleen Lymphocytes Binding Various Rabbit Antisera under "B Cell Conditions"\**

Source of Cell	Diameter of cell $\mu$	Per cent cells labeled†					
		NRS	anti- $\kappa$	anti- $\mu$	anti- $\gamma_1$	anti- $\gamma_2$	anti- $\alpha$
Normal SPF spleen	<6.5	0	6.7	6.2	0	0	0
	6.6-8	0	39.5	41	0	8.2	15
	8.1-10	0	50	37	11	11.5	22
	10.1-12.5	0	40	55	6.7	33	31.5
	>12.5	0	40	45	20	43	29
All Cells		0	37	35	2	8	16
5 day 1° SRBC immunized spleen from SPF mice	<6.5	0	0	0	0	0	0
	6.6-8	7	35	39	0	17.6	
	8.1-10	0	59	43	4	25	
	10.1-12.5	0	71	41	23.4	34.6	
	>12.5	4.7	36	29	28.6	22.8	
All cells		1	52	43	10	18	

\* 1:1000 rabbit antiserum; 1  $\mu\text{g}/\text{ml}$  SARG- $^{125}\text{I}$ ; 3 days' exposure.

† 100 cells were scored, then the edges of the smear were scanned for cells >10  $\mu$  as these were rare in the over-all population.

TABLE X  
*Labeling of Thymus Lymphocytes under T Cell Conditions with Various Antiglobulin Sera\**

No. of serum	Activity of serum	Counts/sec per $10^6$ cells	Per cent lymphocytes labeled		
			3 day exp.	10 day exp.	60 day exp.
Nil	Nil	8.0	1.0	2.7	2.3
R 348	NRS	13.6	1.0	2.0	4.3
R 14	Anti- $\kappa$	126	52	76	87
R 14-BI	No antiglobulin	50	14	28	85
R 19 NS	Polyvalent antiglobulin, predominantly anti- $\kappa$ and anti- $\mu$	216	98	100	100
R 19 S	Anti- $\mu$	97	92	98	100
R 19 S-BI	No antiglobulin detectable	32	39	53	99

\* Labeling conditions: 1:200 rabbit antisera; 5  $\mu\text{g}/\text{ml}$  SARG- $^{125}\text{I}$ .

proportion of cells labeled with anti- $\kappa$  rose progressively. Thirdly, although inspection of smears showed much lower mean grain counts, the blocked reagents still labeled nearly all lymphocytes.

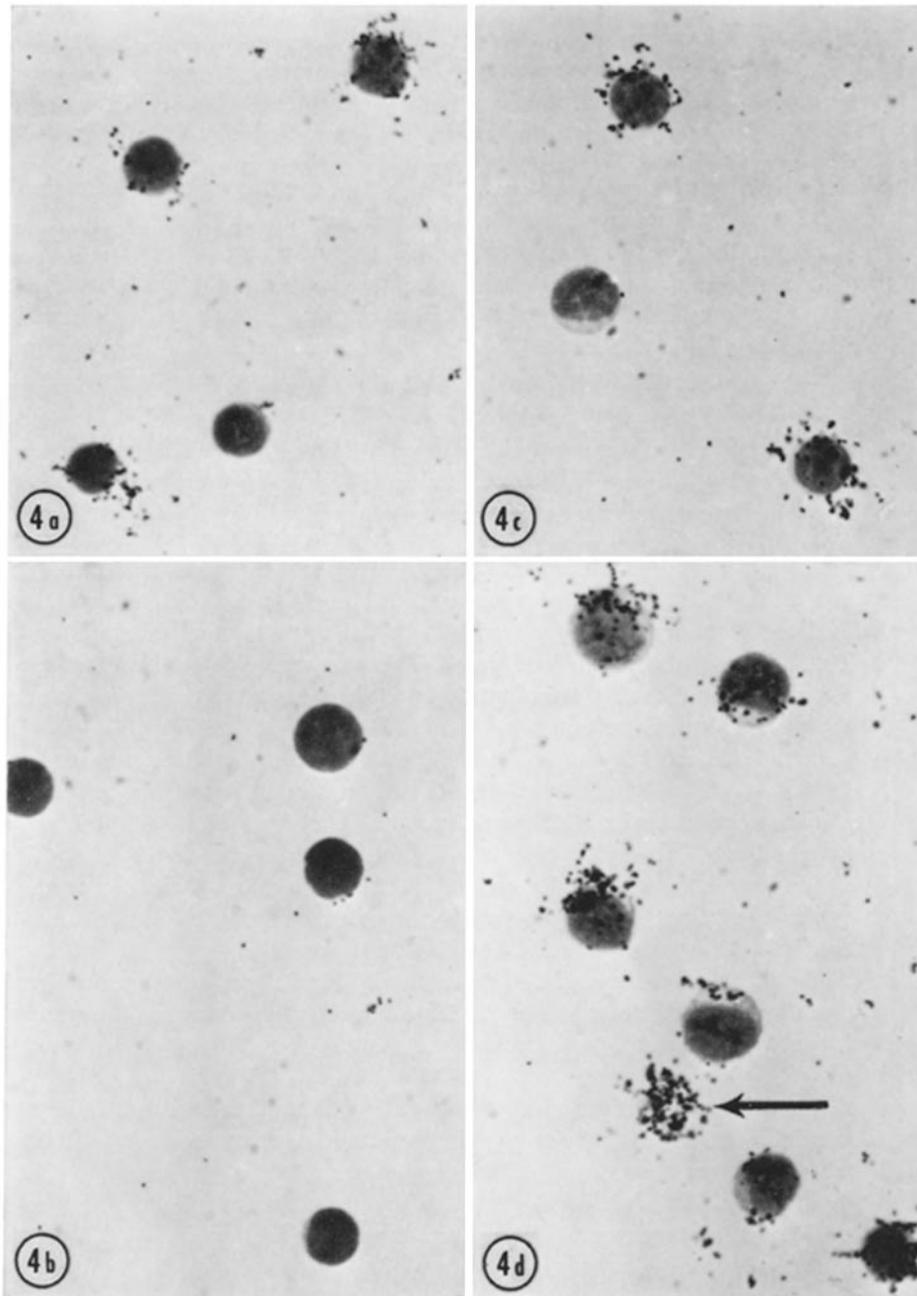


FIG. 4. Photomicrographs of lymphocytes labeled with antiglobulins by the sandwich method. (a) Thymus cells labeled with anti- $\kappa$  serum R 14 under T cell conditions. (b) The same cell suspension labeled with R 14 that had been "blocked" by absorption with mouse light chains coupled to PAPS. (c) Normal spleen cells labeled with R 14 under B cell conditions. Note clear distinction between two labeled and one unlabeled cells. (d) T-TDL labeled under T cell conditions with R 19 NS. Note relatively uniform labeling, and one heavily labeled erythrocyte (arrowed).

No specific binding could be observed with anti- $\alpha$ , anti- $\lambda$ , anti- $\gamma_1$ , or anti- $\gamma_2$  reagents. In several experiments, an anti- $\alpha$  serum gave marginally more binding than NRS but this proved to be a finding that could not be repeated at will. Furthermore, not every batch of specific anti- $\mu$  serum bound significantly. The most consistent results were obtained with polyvalent or anti- $\kappa$  sera.

In many experiments, the grain count frequency distribution over thymus cells was determined. In contrast to the situation in spleen, where a bimodal distribution had been found (Fig. 3), with thymus cells the distribution was unimodal using either polyvalent, anti- $\kappa$  or anti- $\mu$  reagents. When conditions were chosen such that the median grain count was around 20 grains/cell, only about 5% of cells showed <5 grains and 5% over 50 grains, some 75% of cells showing 5–30 grains. Thus the labeling intensity was less heterogeneous than for splenic B cells labeled under B cell conditions. With both thymus cells and T-TDL, the small minority of very big (>15  $\mu$  diameter) cells labeled the most heavily, but as these cells were relatively infrequent, their heavier labeling did not greatly affect the mean grain count. The average size of the small-to-medium lymphocytes was greater for T-TDL than for thymus or indeed for the T cells of normal thoracic duct lymph. This was largely due to increased cytoplasm. However, the T-TDL of median size did not label more heavily than the thymocyte of median size.

In the smears of thymus cells or T-TDL, occasional erythrocytes were encountered. It was of interest that these displayed labeling with specific antiglobulins (but not with NRS) of about equal intensity to that of thymic small lymphocytes. This erythrocyte labeling could not be removed by absorbing either the rabbit antiglobulin and/or SARG repeatedly with mouse erythrocytes. In contrast to thymocytes, erythrocytes labeled with anti- $\gamma_2$  sera to approximately the same extent as with anti- $\mu$  sera.

The following entities labeled more heavily than thymus cells under T cell conditions: dead cells, small fragments of cytoplasm, platelets (encountered frequently in spleen), polymorphonuclear leukocytes, monocytes, and macrophages.

Extensive efforts were made to absorb rabbit antisera with a variety of murine cells such as kidney cells or tissue cultured mastocytoma cells, and these failed to lower the thymus cell-binding power of antiglobulin sera or NRS significantly. When sera that bound to thymocytes were reacted with PAPS coupled with an irrelevant antigen, such as polymerized flagellin, no nonspecific lowering of binding capacity was observed.

*Comparison of Antiglobulin-Binding Capacity of Thymocytes vs. Peripheral T Cells.*—When spleen cells or TDL were reacted under “thymus conditions” with polyvalent antiglobulin or anti- $\kappa$  sera, those cells that had failed to label under B cell conditions virtually all became labeled. Accurate grain counting was rendered difficult by the extraordinarily high degree of labeling of B cells present in the smear, and the consequent high background. Nevertheless, the impres-

sion was formed that the labeling of T cells in the periphery was of the same order of intensity as that of thymocytes.

A more satisfactory approach to this question was to compare two populations of pure  $\theta$  positive cells directly, namely thymus cells and T-TDL. A typical experiment on this point is shown in Table XI. As judged either by bulk scintillation counting or by grain counts, the T-TDL, far from displaying more "receptors" on their surface, actually showed no difference in labeling with the polyvalent antiglobulin and less labeling with the anti- $\mu$  reagent.

The table makes the further point that it is vital to include NRS controls in every experiment. In this experiment, the NRS R 348 (which happened to be

TABLE XI  
*Comparative Labeling\* of Thymus Lymphocytes and T-TDL†*

No. of serum	Activity of serum	Thymus		T-TDL†	
		Counts/sec per 10 <sup>6</sup> cells	Mean grain count‡	Counts/sec per 10 <sup>6</sup> cells	Mean grain count
Nil	Nil	4.8	0.5	2.6	1.8
R 348	NRS	16.8	3.6	13.1	8.0
R 19 NS	Polyvalent antiglobulin predominantly anti- $\kappa$ and anti- $\mu$	95.2	60	106	54
R 19 S	Anti- $\mu$	46.4	32.5	23.6	17.5
R 19 S-B1	No antiglobulin detectable	19.2	8.1	9.9	4.5

\* Labeling conditions: 1:200 rabbit serum; 5  $\mu$ g/ml SARG-<sup>125</sup>I.

† T lymphocytes with killer activity against *H-2* antigens. See footnote § to Table VI.

‡ Adjusted to 5 days' exposure.

|| High NRS control values. See text.

that NRS in our collection which bound most strongly to thymus cells) is beginning to give an annoying degree of labeling of T-TDL. While there is no doubt that R 19 NS labeled T-TDL much more heavily than NRS, the specificity ratio with the anti- $\mu$  reagent R 19 S was only 2, which we consider on the borderline of significance. In the future development of this work, we intend to preselect rabbits on the basis of low binding of their serum to thymocytes, and to use preimmunization serum samples as controls in all cases.

#### DISCUSSION

Radioautography is a technique of extraordinary sensitivity. When pushed to its limit, it can detect 10 molecules of a radioactive substance in or on a cell (25). Potentially, it is 10,000–100,000 times more sensitive than immunofluorescence. While this may confer some advantages, it also imposes a need for extreme concern about specificity and for caution in interpretation. This will become obvious when we consider the question of T cell receptors.

In this paper, we have described a sandwich technique in which rabbit anti-

mouse globulin sera are bound to the surface of lymphocytes and are then exposed to a sheep anti-rabbit globulin reagent labeled with  $^{125}\text{I}$ . The amount of the first reagent bound determines the amount of the second reagent bound at a given molarity, as judged by a large series of preliminary experiments. Thus the technique affords the possibility of comparing, directly and conveniently, the binding strength of a variety of rabbit antisera. There is a considerable logistic advantage in having to purify and radioiodinate only one sheep globulin reagent rather than dozens of rabbit sera, particularly when it is recalled that the mouse myeloma-absorbed rabbit sera are rather precious, and that any IgG isolation procedure for small volumes of serum involves yield losses. Moreover, for cells which bind only small amounts of the first reagent, the sandwich method gives significantly better "signal-to-noise" ratios than does the direct method, as judged by bulk scintillation counting of washed lymphocyte suspensions. For analyses relying purely on radioautography, we found little difference between the direct and the sandwich method, and an eventual choice between the two would depend as much on convenience as any other factor.

As regards B cells, the present work has confirmed the findings of others (3-8) on the capacity of the surface of such lymphocytes to bind antiglobulin reagents under conditions that do not label T cells. The components that can most readily be detected on the B cell surface are  $\kappa$ - and  $\mu$ -chains. However, anti- $\alpha$  and anti- $\gamma_2$  reagents also labeled significant numbers of B cells under conditions where T cells remained completely unlabeled. The percentage of B cells labeling with anti- $\kappa$  was virtually equal to that labeled with polyvalent antiglobulin, and that labeled with anti- $\mu$  was only slightly lower, suggesting that virtually all mouse B cells display  $\kappa$ - and  $\mu$ -chains, as has been reported by Bankhurst and Warner (7). This agrees with a conclusion reached by Pernis et al. for the rabbit (4).

Under equally gentle conditions, a proportion of presumably the same cells labeled with anti- $\alpha$  or, less frequently, with anti- $\gamma_2$ . At face value, this suggested the presence of two, or conceivably even three, heavy chain classes on the surface of some B lymphocytes. This conclusion has been reached on the basis of other evidence also by Sell (23) and by Greaves (9), and confirms the earlier finding of Bankhurst and Warner (7) with the direct radioautographic technique. It stands in contrast with the findings of Pernis et al. (4) with immunofluorescence and of Rabbellino et al. (6) with direct radioautography. The much lighter labeling of cells with anti- $\alpha$  and anti- $\gamma_2$  than with anti- $\mu$ , suggests a smaller amount of  $\alpha$ - and  $\gamma_2$ -chains on the lymphocyte surface, and, as Pernis has suggested, the finding may have a trivial explanation in that it could reflect passive adsorption of serum globulin on to the B cell surface rather than multiclass receptors for antigen. One of us (N.L.W.) has discussed other theoretical possibilities and further experimental approaches extensively elsewhere (26).

The new finding of the present work is that virtually all T cells, be these in

the thymus or at the periphery, can be labeled by antiglobulin reagents, though under conditions involving much higher concentrations of labeling reagents than are needed for B cells. Again, though not all sera of a given specificity react equally well, the evidence favors the presence of  $\kappa$ - and  $\mu$ -chains at the T cell surface, the former being much easier to demonstrate than the latter. This work extends the previous work of Bankhurst et al. (15) using the direct technique. The antiglobulin binding capacity of T lymphocytes is 100–400 times less than that of B cells. Though such factors depend on too many variables to reflect exactly the relative numbers of surface receptors in the two types of lymphocytes, they are consistent with a big difference in receptor density. The strength of binding of antiglobulin reagents to thymocytes did not exceed that of normal rabbit serum by a very large factor. However, specificity was assured (*a*) by the very significant, though incomplete, blocking of the binding through removal of antiglobulin antibodies, (*b*) by a fair correlation between antiglobulin (predominantly anti- $\kappa$ ) activity and thymus-binding activity of a large number of immune rabbit sera, and (*c*) by control absorptions that showed the binding not to be due to anti-lymphocyte-type antibodies. The presence of some immunoglobulin on the surface of thymus cells thus seems clear. We could find no significant difference in the amount of surface immunoglobulin on thymus cells vs. on peripheral T cells or activated T-TDL. The residual labeling of T cells by antiglobulin sera that appeared to be completely blocked with respect to their capacity to bind mouse globulin in the Herzenberg-Warner (16) test warrants comment. This was noted with both B and T cells. It could imply the presence on the surface of lymphocytes of idiotypic, conformational, or V gene subgroup antigenic determinants not represented on the absorbing myeloma. Alternatively, it could mean that the radioautographic method was an even more sensitive means of detecting residual antiglobulin activity than the radioimmunoprecipitation method.

We must now ask whether the presence of a small amount of Ig, perhaps of the order of 500 molecules/cell, on the surface of T cells has any functional significance for the immune response. Are these molecules the antigen receptors? The present results cannot answer this question, but they have given some further weight to the need for caution, as it was shown that even erythrocytes bind approximately as much antiglobulin as do T cells. It may be well to recall that Najjar has shown many cells to have small amounts of immunoglobulin on their surface, without it being implied that these function as receptors (27). Certainly the relatively poor binding of antiglobulins by T cells stands in striking contrast to the strong binding by B cells. On the other hand, it has been shown that antigen-induced functional activation of T cells can be inhibited by anti- $\kappa$  sera (reviewed in reference 26); and that the specificity of interaction of T cells with antigen has much in common with the specificity of serological reactions (28). One possibility that should be seriously entertained is that the T cell receptor for antigen is indeed a different class of immunoglobulin (IgX),

that cross-reacts more with IgM than with any other class of serum antibody. Another is that only the V regions of  $\kappa$ - and  $\mu$ -chains are exposed on the T cell surface; as these are relatively poorly immunogenic, anti- $\kappa$  or anti- $\mu$  antisera would have only few molecules directed against them. This idea has been explored by Greaves (9), but we have been unable to obtain evidence for his claim that progressively more of the IgM molecule is exposed on T cells displaying increasing activation. The concept that the T cell receptor is some totally different kind of molecule has also not been ruled out by the present experiments.

The chief value of the present results is threefold. First, a simple and convenient method has been devised to allow the screening of many sera with putative anti-T cell receptor antibody activity. Secondly, immunoglobulin has been positively identified on the surface of all T cells, but under much higher concentrations of the binding reagents. Thirdly, a quantitative sandwich method of potential value for the study of receptor metabolism has been characterized. Our next paper will document its usefulness in this last respect.

#### SUMMARY

The present study was designed to devise and characterize an indirect or sandwich radioimmunolabeling technique for the study of lymphocyte surface receptors of immunoglobulin nature. Mouse lymphocytes from various sources were treated by the method of Shortman et al.<sup>3</sup> to remove debris and damaged cells. This was an important preliminary step, as without it, little meaning could be attached to bulk scintillation counting of labeled cell suspensions, in view of the marked tendency of dead or damaged cells to adsorb protein nonspecifically. Next, cells were reacted at 0°C for 30 min with graded dilutions of unlabeled rabbit antisera against defined mouse Ig chains. After washing, the cells were reacted with a sheep anti-rabbit globulin reagent labeled with <sup>125</sup>I, again at graded concentrations. After further washing, lymphocyte labeling was quantitated by both bulk scintillation counting and radioautography.

Conditions were defined in which nonthymus-derived cells (B cells) but not thymus-derived cells (T cells) could be labeled. Most B cells displayed  $\kappa$ - and  $\mu$ -chains on their surface, but some also displayed  $\alpha$ - and  $\gamma_2$ -chains, though in smaller amounts. When the concentration of both the first and the second reagents were raised considerably, conditions were defined under which virtually all T cells could be labeled by polyvalent antiglobulin sera, anti- $\kappa$  sera, or, with more difficulty, by anti- $\mu$  sera. A large series of control experiments confirmed the serologic specificity of this labeling. It was shown that under equivalent conditions, B cells bind 100-400 times more antiglobulin than do T cells.

The theoretical implications of the results are briefly discussed. It is argued that the sandwich approach offers certain technical advantages over direct labeling procedures for further analyses of T cell receptors and for studies of receptor metabolism.

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