IMMUNOGLOBULIN SPOTS ON THE SURFACE OF RABBIT LYMPHOCYTES*‡

By BENVENUTO PERNIS, M.D., LUCIANA FORNI, AND LUISA AMANTE§

(*From the Laboratory of Immunology, Clinica del Lavoro, University of Milan, Milan, Italy and the Clinica del Lavoro, University of Genoa, Genoa, Italy*)

There is considerable evidence for the presence of immunoglobulins on the surface of lymphocytes in different species. This evidence is given by the transformation of rabbit lymphocytes into immunoblasts after contact with anti-immunoglobulin or anti-allotypic antisera (1), formation of rosettes with the same antisera in the presence of sensitized erythrocytes (2), and inhibition of rosette formation (3). Raff et al. have recently reported the presence of immunoglobulins on mouse lymphocytes as detected by immunofluorescence and autoradiography (4).

The work described here shows that about one half of peripheral blood and spleen lymphocytes in the rabbit carry on their surface a number of discrete immunoglobulin spots, and that the immunoglobulins present on a single cell are uniform with regard to the allotype. It appears that the immunoglobulins found on the lymphocyte surface are the product of the same cell that carries them, and that they are firmly attached to the cell membrane.

These findings have been obtained by immunofluorescent staining of living cells in suspension.

Materials and Methods

Animals.—Normal (nonimmunized) adult rabbits were used in all the experiments for the detection of immunoglobulin determinants. Young rabbits were used for the study of thymus lymphocytes.

For the study of antigen-reactive cells, rabbits received 4 mg horse ferritin (Mann Research Labs., New York) (recrystallized) in complete Freund's adjuvant, and a second injection in incomplete adjuvant 1 month later. Several weeks after the second injection peripheral blood lymphocytes were prepared and processed as indicated below. The animals were then restimulated with 3 mg of soluble horse ferritin in the hind footpads; popliteal lymph nodes were taken on the 5th and 6th day after the booster injection.

Antisera

Anti-Allotypic Antisera.—Antisera to *a* and *b* locus allotypic determinants as well as an antiserum to the IgM-specific allotype Ms3 (5), prepared as described by Kelus and Gell (6)

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were kindly supplied by Dr. A. S. Kelus. Several antisera were screened for specificity not only in the usual agar double diffusion technique but also by checking the reactivity of the fluorochrome-conjugated antisera on plasma cell smears prepared from rabbits of known genotype. In this way it became apparent that a fair number of antisera contained additional anti-allotypic antibodies, particularly directed against some hitherto unknown IgM allotypes.[1] These antisera were not used for the present investigation.

Anti-As11 and anti-As12 antisera (7–8) were obtained through the courtesy of Doctors C. W. Todd and W. J. Mandy.

Specific Anti-IgM Antiserum.—A sheep was immunized with sheep red cell stromata covered with rabbit antibodies to sheep red blood cells (SRBC)[2] (serum was taken at the 4th day after a single injection of SRBC). This antiserum contained antibodies to rabbit IgM and some antibodies against complement components. To make it specific for rabbit IgM, the antiserum was absorbed with newborn rabbit serum made insoluble with ethyl chloroformate (9) and with zymosan particles covered with rabbit complement components (mainly $\beta_1 C$) as described by Mardiney and Müller Eberhard (10). The absorbed antiserum gave a single line in the beta region when tested by immunoelectrophoresis against whole rabbit serum, and did not agglutinate tanned SRBC coated with purified rabbit IgG or IgA.

Specific Anti-IgG Antiserum.—A sheep was immunized with the Fc fragment prepared from commercially available rabbit gamma globulins (Cohn FII, Mann Research Labs.) by the method of Porter (11). This antiserum, however, contained some antibodies to Fab fragment, and was therefore absorbed with pepsin (Fab)$_2$ fragment (12) made insoluble by cross-linking with ethylchloroformate (9). The absorbed antiserum did not react in double diffusion with purified rabbit light chains, and did not agglutinate tanned SRBC coated with rabbit IgA.

Anti-Ferritin Antiserum.—This antiserum was prepared in rabbits using an immunization schedule as described above. Serum was taken 1 week after the second injection of horse ferritin.

Conjugation of Antisera.—The antisera were conjugated with fluorochromes by the method of Cebra and Goldstein (13) modified by Amante and Giuriani (14). The antisera were diluted with an equal volume of saline (0.15 M NaCl) and solid ammonium sulfate added to reach a concentration of 1.6 M. The precipitate was washed in 2 M ammonium sulfate, resuspended in phosphate buffer 0.01 M pH 7.5, and dialyzed against the same buffer until free of sulfate ions. The globulin solution was then applied to a DEAE-cellulose column and eluted stepwise with 0.01 M phosphate buffer pH 7.5 (basic fraction) and the same buffer containing 0.05 M NaCl (acid fraction). With sheep antisera two peaks of basic globulins were obtained; both were found suitable for conjugation with fluorescein isothiocyanate. Different amounts of fluorochromes (fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate, Baltimore Biochemical Labs. Baltimore, Md.) per milligram of protein were used in different conditions according to the amount of protein available; this was found necessary to obtain the optimal protein–fluorochrome ratio of the conjugates (14). The conjugation and purification of conjugates by DEAE-cellulose chromatography were carried out as described by Cebra and Goldstein (13). The protein–fluorochrome ratio was calculated on the basis of the optical density at 280 nm (for protein concentration) and at 495 and 515 nm (for fluorescein and rhodamine, respectively). Only eluates with conjugation values between 2 and 3 were used. The final protein concentration was arranged at 0.5 mg/ml. Normal serum (3% v/v) of the same species and allotypic specificity of the antiserum donor (sheep or rabbit) was added to the conjugates. These were then sterilized by Millipore filtration (Millipore Filter Corp., Bedford, Mass.) and stored at 4°C.

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[1] Kelus, A. S., and B. Pernis. Manuscript in preparation.

[2] *Abbreviations used in this paper:* BSA, bovine serum albumin; BSS, balanced salt solution; PBS, phosphate-buffered saline; SRBC, sheep red blood cells.

Preparation of Lymphocytes

Peripheral Blood Lymphocytes.—Blood was obtained from the heart of the rabbits with a heparinized syringe and collected in a heparinized bottle. One-half volume of 3% (w/v) pigskin gelatin (Eastman-Kodak Co., Rochester, N.Y.) in Hanks' balanced salt solution (BSS) was added to the blood and the mixture was allowed to sediment for 1 hr at 37°C. The leukocyte-rich supernatant was collected, centrifuged at 1000 rpm for 15 min and the cells washed once with warm Hanks' BSS. The red cells were eliminated by washing the cells once or twice at room temperature with a buffered ammonium chloride solution (0.75% NH_4Cl in 0.02 M Tris, pH 7.65; final pH of the solution 7.2). Finally the granulocytes were bound on a small nylon column at 37°C for 30 min, and the cells eluted with warm Hanks' BSS. The lymphocytes in the final suspension were counted and resuspended in phosphate buffered saline (PBS) (pH 7.2) containing 5% (w/v) bovine serum albumin (BSA) at a concentration of 15–20 million cells/ml.

Spleen, Lymph Node, and Thymus Cells.—The tissues were cut in small pieces and a cell suspension was made with a loose-fitting glass homogenizer in cold BSA-PBS and filtered through a 400 mesh stainless steel gauze. The entire procedure was carried out in an ice-bath. After allowing spontaneous sedimentation of cell clumps, the suspension was washed twice in the cold with BSA-PBS. The cells were counted and the suspension adjusted at 15–20 million cells/ml. In some cases cell smears were prepared with a cytocentrifuge (Shandon-Elliott, London, England).

Staining of Cells.—The immunofluorescent staining of cells was performed by mixing equal volumes of the cell suspension with the appropriate antiserum or mixture of antisera precooled in an ice-bath. The mixture was kept for 30 min in an ice-bath, then the cells were washed three times in the cold with BSA-PBS. Finally the cells were resuspended in one drop of the same solution or in phosphate buffered glycerol, pH 7.2 (there was no difference in preparations mounted in the two different ways), put on slides, covered with cover slips, and sealed with nail polish. The preparations were then viewed immediately, as described below. In some experiments, after staining in suspension as described above, the cells were flattened on slides through the use of a cytocentrifuge, fixed for 5 min with ethanol, rehydrated in PBS, and mounted in phosphate-buffered glycerol. This procedure, similar in principle to that of Cerottini and Brunner (15), permits a good reaction of the surface antigens with the antisera and there is no subsequent loss of localization of conjugated antibody. Since the cells treated in this way are flattened on the slide, the counting of immunoglobulin spots is considerably facilitated; the spots are also much easier to demonstrate by photography than those on spherical cells where only one part of the surface can be focused on at a given time.

To establish the influence of the concentration of conjugated antisera on the percentage of lymphocytes stained, an experiment was done with a rhodamine-conjugated anti-As4 antiserum diluted in various proportions with BSA-PBS.

Double staining for allelic allotypes, as well as double staining for *a*- and *b*-locus allotypes, was performed by incubating the cells with a mixture of equal amounts of the two antisera. In addition, to collect some information on the limits of detection of a minor component of a conjugated antiserum in the presence of a major amount of the other fluorochrome, peripheral blood lymphocytes from an As4,4 homozygous rabbit were stained with different mixtures of fluorescein- and rhodamine-conjugated anti-As4 antiserum. The proportions of the rhodamine-conjugated component were 25, 10, 5, 2.5, and 1% of the mixture. The rhodamine conjugate was still detected, although only on the cells which were more rich in surface immunoglobulins, with a mixture composed of 97.5% fluorescein-conjugated and 2.5% rhodamine-conjugated anti-As4.

For the detection of As11 and As12 determinants, a sandwich technique was used, first involving mixing the cells with the unconjugated antisera and then detecting anti-As11 and

anti-As12 antibodies (both made in As1/4 rabbits) with a rhodamine-conjugated anti-As1. This staining technique was of course applied only to cells from non-As1 rabbits.

For the detection of antigen-reactive cells, the cell suspensions from both immunized and nonimmunized rabbits were incubated with horse ferritin. Different concentrations of the antigen were tested; the percentage of reacting cells increased up to a concentration of 2 μg/ml after which a plateau was obtained. A concentration of 10 μg/ml was therefore routinely used. The cells were exposed to the antigen for 30 min in an ice-bath and then washed three times with BSA-PBS in the cold. The pellet was then resuspended, mixed with an equal volume of rhodamine-conjugated anti-ferritin antiserum, and processed as usual.

Microscopy.—The preparations were examined with a Leitz ortholux microscope (E. Leitz, GMBH, Wetzlar, Germany) equipped with an Osram HBO-200 mercury vapor lamp and an Opak-Fluor vertical illuminator; this illuminator and appropriate combinations of exciting filters, dichroic mirrors, and barrier filters allows the selective visualization of fluorescein or rhodamine (16). The use of this optical system resulted in a major improvement both with regard to the possibility of detecting only one of the two fluorochromes with absolute selectivity and with regard to the intensity of fluorescence. In fact the sensitivity of detection of the fluorescent staining was very much enhanced, particularly with the rhodamine-conjugated antisera.

Each microscopic field was observed alternatively in phase contrast to count all the cells, and in specific illumination to count the stained cells. The following combinations of exciting and barrier filters and of dichroic mirrors were used: (*a*) for visualization of both rhodamine and fluorescein, exciting filter 4 mm BG12, dichroic mirror 495 nm, barrier filters K 495 plus K 530; (*b*) for selective visualization of fluorescein, exciting filter 4 mm BG12, dichroic mirror 495 nm, barrier filters K 495 plus AL 530; (*c*) for selective visualization of rhodamine, exciting filters 4 mm BG38 plus 2 mm BG 36 plus AL 546, dichroic mirror 580 nm, barrier filters K 580 plus K 590. Photographs were recorded on Ektachrome high speed daylight 23 DIN film, or on Kodak TRI-X Pan 27 DIN Film.

Treatment of Cell Suspensions with Sodium Azide.—Cell suspensions from As4,4 homozygous rabbits were stained with rhodamine-conjugated anti-As4 antiserum in the presence of 1.5 $\times$ 10^{-2} M sodium azide.

Controls.—Three kinds of controls were performed: (*a*) Cell suspensions were treated with unrelated conjugated antisera (e.g. anti-As1 on cells from As3,3 rabbits). No positive cells were seen in preparations from different animals stained with different antisera. (*b*) Cell suspensions were treated with undiluted unconjugated antisera, washed, and exposed to the same antisera labeled with rhodamine. A complete and specific inhibition of the staining was observed. (*c*) In experiments on antigen-reactive cells, peripheral blood and lymph node lymphocytes were treated with rhodamine-conjugated anti-ferritin antiserum without pretreatment with ferritin. No fluorescent cells were seen in any preparation from either immunized or nonimmunized animals.

RESULTS

Detection of Immunoglobulin Determinants on Rabbit Lymphocytes.—Various aspects of the lymphocytes of an As4,4 rabbit, after staining with a conjugated anti-As4 antiserum, are shown in Figs. 1–3. About one-half of the cells, either from the peripheral blood or the spleen, appeared covered with numerous fluorescent spots; some cells had numerous faint small spots, some had a fair number of small bright spots, others had a few large bright patches, and many others had different combinations of the above patterns. As an average, more

than 50 spots could be identified on a single cell. From the planimetry of en-
larged photographs it could be calculated that, again as an average, about
one-fourth of the surface of the positive cells was covered by immunoglobulins.
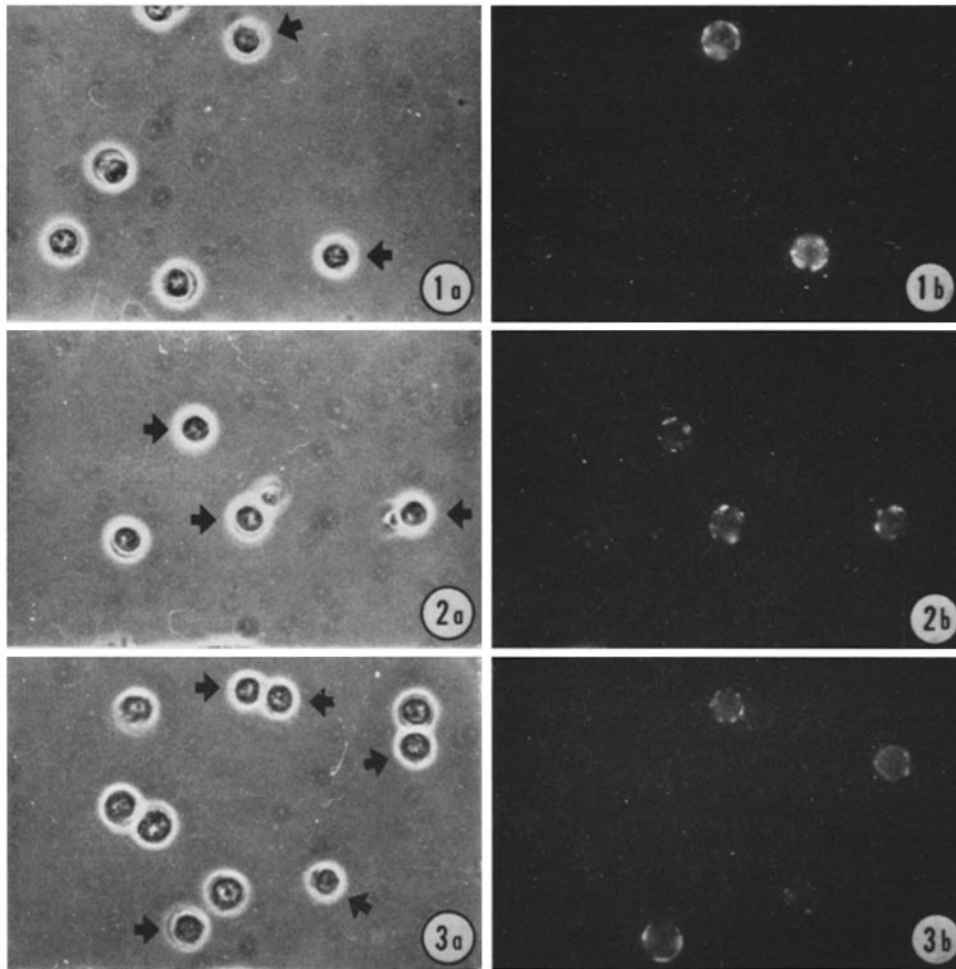
FIGS. 1–3. Rabbit As4,4. Peripheral blood lymphocytes stained in suspension with
rhodamine-conjugated anti-As 4. × 540. (a) Phase contrast, (b) selective visualization of
rhodamine. The positive cells correspond to those marked by arrows in the phase contrast
pictures.

The fluorescent spots were distributed as a rule on the entire surface of the
cells. In some cells, the fluorescent spots were hardly discernible and, instead,
an almost continuous fluorescence of the membrane (that is a ring appearance)

was seen. These cells were usually quite scanty, and in some preparations they were absent; the possibility that the ring appearance is connected with damage to the cell is presently being investigated.

When viewing the cells under high power ($\times$ 540–1000) it was never possible to have in focus all the fluorescent spots of a single cell. In fact, focusing up and down, the impression of the distribution of the spots on the surface of a sphere was always obtained. When the stained cells were flattened on slides by centrifugation, they appeared to be covered by fluorescent spots (Fig. 4).

Essentially the same picture was observed with cells stained with anti-Ms3 (a IgM-specific allotypic marker [5]) and with a specific anti-IgM. On the other
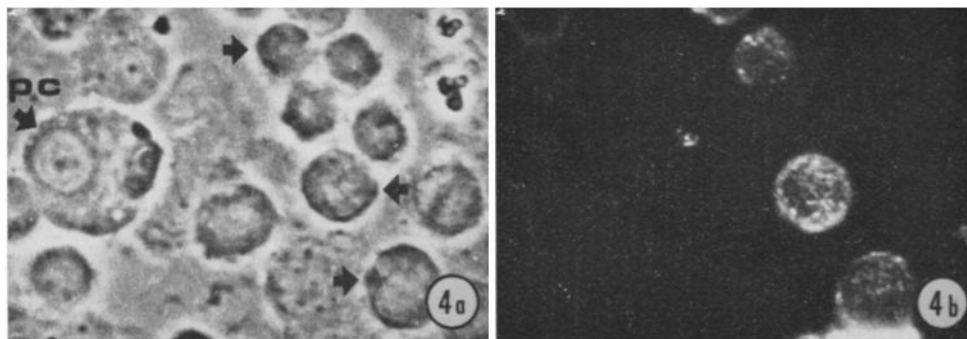

Fig. 4. Rabbit As4,4. Spleen cells stained in suspension with rhodamine conjugated anti-As4 and flattened afterwards on a slide by centrifugation. Lymphocytes and medium lymphocytes are visible in phase contrast (a) showing fluorescent spots under specific illumination for rhodamine (b). A plasma cell (pc) is visible, lacking fluorescent surface staining. $\times$ 1000.

hand the cells stained with anti-As 11 and anti-As 12 (IgG-specific allotypic markers [7, 8]) showed a much lower average number of fluorescent spots per cell; the spots were also smaller in this case.

All the preparations were viewed alternatively with the illumination for fluorescence and in phase contrast; it was thus possible to ascertain that the cells with fluorescent spots had the appearance of small and medium lymphocytes. In the preparations obtained from the spleen there were always a certain number of very large cells, presumably immunoblasts, that had fluorescent spots on their surface. On the other hand many cells were seen with the aspect of mature plasma cells; these cells did not show surface staining (Fig. 4). This point is being further investigated.

It must be stressed that in all preparations a number of small and medium lymphocytes were found which apparently did not react at all with fluorescent antisera; in phase contrast these cells could not be distinguished in any way from the positive ones.

The percentage of positive cells as well as their appearance with regard to the number or size of fluorescent spots did not change when the staining was performed in the presence of sodium azide, an agent which is known to effectively inhibit pinocytosis (Table I).

The influence of the concentration of the antiserum on the percentage of peripheral blood lymphocytes of an As4,4 rabbit that were stained by a rhoda-

TABLE I

Percentage of Cells Stained by a Conjugated Anti-As4 Antiserum in the Presence or Absence of Sodium Azide

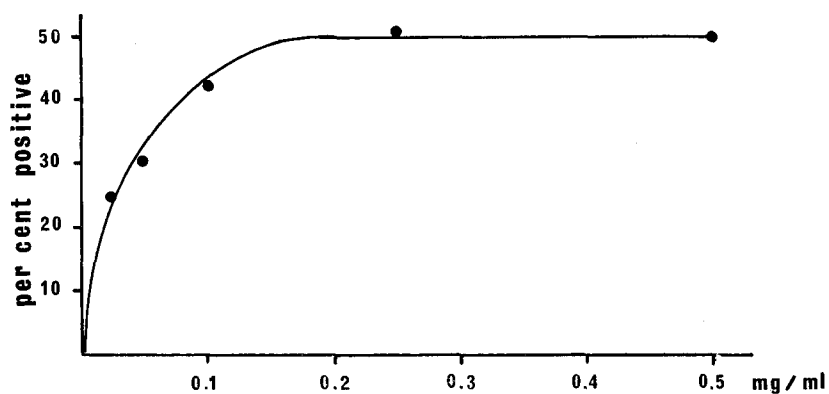| Rabbit No.	Rabbit allotype	Treatment	Positive lymphocytes as per cent of total lymphocytes
R-201	As 1,3/4,4	Sodium azide	38
		None	36
R-218	As 1,1/4,4	Sodium azide	41
		None	42

FIG. 5. Percentage of cells stained in suspension by an anti-As4 antiserum as function of antiserum concentration.

mine-conjugated anti-As4 antiserum is given in Fig. 5; it is apparent that a plateau was already reached with concentrations of the antiserum that were one-third of those routinely used in our experiments. This test, which gives confidence to the assumption that the percentage of stained cells does not depend on the concentration of the conjugated antiserum used for staining, was not, however, repeated for all the antisera used.

The percentage of lymphocytes reacting with antisera directed against different allotypic determinants of rabbit immunoglobulins is given in Table II. The data are given as positive cells per 100 lymphocytes. In the peripheral blood

preparations practically all the cells were lymphocytes, whereas in spleen preparation, this was not the case; in the latter instance the "nonlymphocytes," as far as they could be recognized under phase contrast, were excluded from the counts.

It is apparent from Table II that very similar results are obtained from different animals, and that moreover, spleen and peripheral blood lymphocytes do not differ much with regard to their percentage of immunoglobulin-positive cells. The lymphocytes with As4 determinants in As4,4 rabbits range from 40 to 50%, with a mean value of 44%.

It is also apparent from Table II that the majority of the lymphocytes, both in the spleen and the peripheral blood, carry allotypic determinants (Ms3) as-

TABLE II

Percentage of Lymphocytes with IgM-Specific and IgG-Specific Allotypic Determinants in Spleen and Peripheral Blood of Rabbits of Different Allotypes

Rabbit No.	Rabbit allotype	Source of lymphocytes	Lymphocytes reacting in suspension with anti-allotypic antisera as per cent of total lymphocytes			
			As 12	As 11	Ms 3	As 4
RB-8251	As3,3/4,6 Ms3	Spleen	not done	not done	36	not done
RTO-3	As3,3/4,4/11,11/Ms3	"	"	4.4	34	43
R-204	As3,3/4,4/11,11/Ms3	"	"	6.8	38	40
R-219	As3,3/4,4/11,11/Ms3	"	"	4.0	35	not done
R-219	As3,3/4,4/11,11/Ms3	Peripheral blood	"	1.5	32	not done
R-215	As3,3/4,4/11,12/Ms3	"	2.3	4.9	42	47
R-216	As3,3/4,4/11,12/Ms3	"	2.0	3.3	42.5	50
R-208	As3,3/4,4/12,12/Ms3	"	2.1	0	42	40
R-211	As3,3/4,4/12,12/Ms3	"	1.5	0	46	46.5
R-214	As3,3/4,4/12,12/Ms3	"	4.1	0	35	50
R-220	As3,3/4,4/12,12/Ms3	"	2.8	0	38	50

sociated with the IgM class, whereas a small proportion of the positive cells (in the average about one-tenth) show determinants associated with the IgG class. This predominance of lymphocytes with IgM was confirmed in a few experiments in which the lymphocytes were stained with a specific sheep anti-rabbit IgM antiserum; in these experiments the percentage of lymphocytes which reacted with the anti-IgM antiserum corresponded to that of cells which were positive with the anti-Ms3 antiserum.

On the other hand, our specific anti-IgG antiserum did not react with any of the lymphocytes in suspension (although it reacted very well with plasma cells in fixed smears); this apparent discrepancy between the results we have obtained using antisera to IgG allotypes and to IgG isotypes will be discussed later. As a consequence, we were not able to perform a double staining with anti-IgG and anti-IgM to ascertain whether immunoglobulins of these two classes were present on different lymphocytes; a double staining with antisera directed to IgG-specific and IgM-specific allotypic markers also could not be

performed due to the allotypic specificity of the antisera themselves (the donor of anti-Ms3 antiserum was proved to be an As11,12 heterozygote).

When cells from As3,3/4,4 rabbits were stained with a mixture of anti-As3 and anti-As4 conjugated with different fluorochromes, the great majority of positive cells were found to carry both H and L chain determinants; rare cells however were observed which lacked one or the other specificity (3 b negatives and 6 a negatives/100 immunoglobulin-carrying cells).

Detection of Allelic Specificities on Lymphocytes from Heterozygous Rabbits.— With spleen and peripheral blood lymphocytes of seven rabbits which were
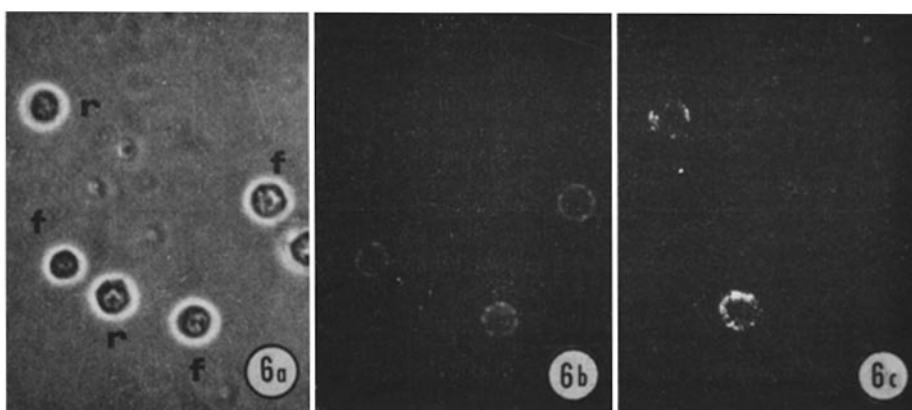

Fig. 6. Rabbit As4,6. Peripheral blood lymphocytes stained in suspension with a mixture of fluorescein-conjugated anti-As4 and rhodamine-conjugated anti-As6. × 540. (a) Phase contrast, (b) selective visualization of fluorescein; three cells are visible which correspond to the cells marked by f in the phase contrast picture, (c) selective visualization of rhodamine; two cells are visible which correspond to those marked by r in the phase contrast picture.

heterozygous at the b locus, and in two heterozygotes at the a locus, double staining experiments were performed in order to investigate whether the products of two allelic genes were located on the same or on different cells; in all experiments a complete differentiation of immunoglobulin-bearing lymphocytes for allelic allotypes was found (Fig. 6).

Counts of the percentage of lymphocytes bearing one or the other of the allelic allotypic specificities were done only for the experiments on b locus markers, and are reported in Table III.

Spleen cells from two of the rabbits were centrifuged on slides, fixed, and stained with the same mixture of antisera used for lymphocytes in suspension; very similar ratios for the two allelic allotypes in lymphocytes and in plasma cells were observed.

The possibility that this apparent allelic exclusion was due to competition

between antisera in reacting with closely interspaced allelic determinants was ruled out by the results obtained by staining the same cell suspension with a mixture of anti-As4 and anti-As6 and with the same antisera separately. In one rabbit (R-181 of Table III) in which 44% positive cells were found with

TABLE III

Percentage of Lymphocytes with Surface Immunoglobulins of One or the Other Allelic Allotypic Specificity in Heterozygous Rabbits

Rabbit No.	Rabbit allotype	Source of lymphocytes	Lymphocytes carrying allotypic markers as per cent of total lymphocytes			As4–As6 ratio	
			As 4	As 6	As 4 + As 6	Lympho-cytes in suspension	Plasma cells in fixed smears
RTO-1	As1,2/4,6	Spleen	18	13	0	58/42	not done
RTO-4	As3,3/4,6	"	25.4	17.6	0	59/41	51/49
RTO-2	As2,3/4,6	"	18.8	14.2	0	57/43	55/45
R-181	As1,3/4,6	"	32.1	11.9	0	73/27	not done
R-199	As1,3/4,6	Peripheral blood	37	20	0	65/35	"
R-230	As1,3/4,6	"	36	24	0	60/40	"
R-235	As1,1/4,6	"	18.9	18.1	0	51/49	"

TABLE IV

Percentage of Cells Showing Specific Surface Binding of Horse Ferritin in Nonimmunized and Immunized Rabbits

Rabbit No.	Immunization*	Source of lymphocytes	Time after the last in-jection of antigen	No. of positive cells‡	Per cent
			(days)		
R-201	None	Peripheral blood	—	2/2700	0.074
R-218	None	"	—	1/1784	0.056
R-214	Horse ferritin I.M.	"	37	10/2332	0.43
R-187	"	"	70	52/5435	0.95
R-175	"	"	83	35/3527	0.99
R-187	Horse ferritin in footpads	Lymph nodes (popliteal)	5	145/1475	9.8
R-175	"	"	6	65/747	8.7

* See Materials and Methods.

‡ Staining done in the presence of 1.5 × 10⁻² m sodium azide.

an As4/As6 ratio of 73/27, 35% positive cells were counted in the sample stained with anti-As4 alone, and 13% positive cells in the sample stained with anti-As6 alone. Preincubation of the cell suspension with unconjugated antisera completely prevented the staining with the conjugated ones.

Detection of Antigen-Reactive Cells.—Ferritin-reactive cells were observed in both immunized and nonimmunized rabbits, although in different proportions. The results are collected in Table IV.

The appearance of cells stained for antibody was very similar to that of cells positive for immunoglobulin determinants (Figs. 7 and 8).

There were no stained cells in the control samples in which exposure to horse ferritin was omitted.
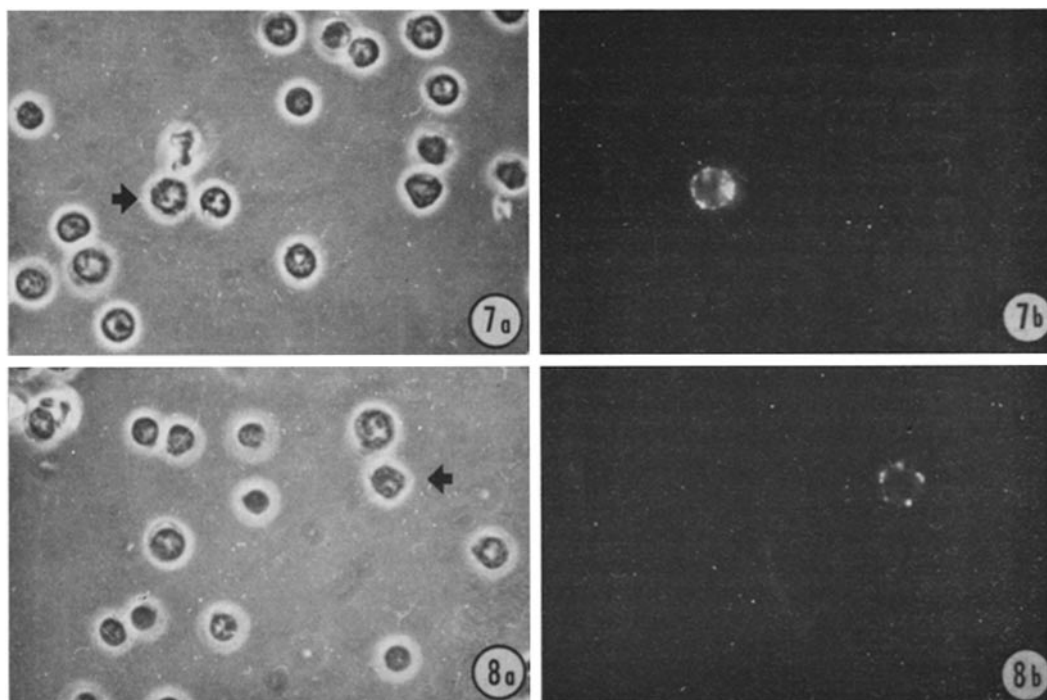
FIGS. 7 and 8. Rabbit immunized with ferritin, 70 days after a secondary injection of antigen. Peripheral blood lymphocytes incubated with ferritin and stained in suspension with a rhodamine-conjugated anti-ferritin antiserum. (a) Phase contrast, (b) selective illumination for rhodamine. Ferritin-binding cells show a pattern of fluorescence similar to that of cells stained with anti-immunoglobulin–allotypes antisera.

Immunoglobulin Determinants on Thymocytes and Bone Marrow Cells.—Mention must be made of the results obtained on the thymus cells. In two young rabbits (2 month), As4 homozygotes at the *b* locus, practically all the thymocytes appeared negative after contact with a conjugated anti-As4, while the same antiserum stained 48 and 49%, respectively, of the spleen lymphocytes of the same rabbits.

A single experiment was done on bone marrow cells; 13% positive cells were found amongst the cells with the appearance of small lymphocytes. It must be noted that in this experiment, done on bone marrow cells from an As4,6 heter-

zygote (R-181 of Table III), complete separation of allelic allotypes was found, with an allelic ratio similar but not identical to that observed in spleen lymphocytes (73/27 in spleen and 84/16 in bone marrow).

DISCUSSION

The observations reported above confirm previous evidence indicating the presence of immunoglobulin determinants on rabbit lymphocytes. This evidence was given by transformation of lymphocytes into immunoblasts after contact with anti-immunoglobulin or anti-allotypic antisera (1), and by the observations performed with the mixed antiglobulin reaction (using sensitized erythrocytes as indicator cells) by Coombs et al. (2). In another species, the mouse, the presence of immunoglobulins on the lymphocytes has been shown recently by Raff et al. through immunofluorescence with a method of vital staining similar to the one used here (4); this result is in agreement with observations already done by Möller (17).

In addition our results show that the lymphocytes are differentiated with regard to the allotype of their immunoglobulin, in the same way as the plasma cells (18). This is at variance with results recently obtained by Wolf and Coombs[3] with the use of the mixed antiglobulin reaction. With this method it appears that, at least in adult rabbits, a variable but sometimes quite sizable proportion of the peripheral blood lymphocytes of heterozygous rabbits does form mixed rosettes with erythrocytes sensitized with one or another of two allelic b locus markers (As4 and As6). We cannot at present give a definitive explanation for this discrepancy. It is possible, however, that the mixed antiglobulin reaction can detect a minor immunoglobulin component on the surface of lymphocytes, below the threshold of about 2.5% that we have estimated to be the limit of immunofluorescence under optimal conditions (see Materials and Methods). If this explanation is valid, the question of the origin of this minor component would still remain open; we think that, dealing with surface immunoglobulins, the passive uptake by some lymphocytes of small amounts of immunoglobulins (as cytophilic antibodies or as antigen—antibody complexes) would be difficult to exclude, and that this alternative would be more likely than the other one of a strongly unbalanced synthesis of allelic allotypes.

Thus we are of the opinion that the principle of allelic exclusion is valid for all the lymphocytes which have surface immunoglobulins detectable by immunofluorescence.

Since it seems that lymphocytes are also differentiated with regard to the class of their immunoglobulins (3) (although we have not been able to demonstrate this directly, for technical reasons), the picture that emerges is that the laws that control the synthesis of immunoglobulins are the same for lympho-

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[3] Wolf, B., and R. R. A. Coombs. Personal communication.

cytes and plasma cells. Furthermore, it appears that both *b* locus and *a* locus markers (as well as class-specific allotypic determinants) are found on the surface of rabbit lymphocytes; this presumably indicates that, whole immunoglobulin molecules and not isolated heavy and light chains are located in that position.

If the relative number of cells differentiated for one or another allelic allotype appears to be very similar for lymphocytes and plasma cells, this does not seem to be the case for the relative frequencies with which cells carrying immunoglobulins of different classes are found amongst plasma cells and lymphocytes. It is in fact apparent from the data of Table II that in all preparations of lymphocytes, from both spleen and peripheral blood, there is a greater number of lymphocytes with markers characteristic of IgM than lymphocytes with specificities indicating the presence of IgG. This is the reverse of what is usually found in plasma cells; in fact IgM-containing plasma cells have been found to be, in different rabbits, 25–40% of total immunoglobulin-producing cells[4] (also Cebra, reference 19).

It is of course attractive to speculate upon this observation, particularly in connection with the possible role of the immunoglobulins of the cell surface as antigen receptors, and the well known prevalence of IgM antibodies in the early stages of the immune response. However it must be pointed out that in a recent paper by Coombs et al. (2) on the immunoglobulins that can be demonstrated on the surface of lymphocytes with the method of mixed antiglobulin reaction, rather variable results have been reported. In some animals, the majority of peripheral blood lymphocytes appeared positive for IgM, but in many others the cells bearing IgG were instead more numerous. This individual variability contrasts with the relative constancy of the IgM—IgG ratios that we have seen so far, and it appears that, before speculating further, one should clarify the origin of this apparent discrepancy.

The immunoglobulin molecules are obviously located on the surface of lymphocytes; this is borne out by the fact that living cells reacted in suspension with the conjugated antisera under conditions in which penetration of antibodies into the interior of the cells did not take place (by pinocytosis or other ways) to any appreciable extent. In fact there is no staining of the immunoglobulins contained in the cytoplasm of plasma cells which were present in spleen-cell suspensions. Furthermore, by focusing up and down, it was easy to appreciate, particularly with the optical system we used, that the immunoglobulin spots were distributed on the more or less spherical surface of the lymphocytes.

The immunoglobulin molecules appear to be connected to the external membrane of the lymphocytes; the bond must be rather stable since the immunoglobulins cannot be removed by repeated washings, and since antigen–antibody

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[4] Personal observations.

layers (see for instance our experiments with ferritin) can be built on them without detaching the immunoglobulins from the surface. The nature of the bond of the immunoglobulins with the membrane components is however unknown, and it is clearly a matter for further investigation.

Whichever the nature of the bond might be, our results, particularly the ready reactivity of lymphocyte immunoglobulins with antisera directed against *a* and *b* locus determinants which are known to be located on the Fab portion of the molecule (6), suggest the possibility that the molecules might be oriented with the Fab outwards. In addition, hinge region determinants are also available for reaction, since we have seen cells stained by anti-allotypic antisera (anti-As 11 and anti-As 12) directed against determinants which are known to be located in that part of the chain (20). The determinants of the IgM-specific allotype Ms 3 are also probably located at the level of the junction between light and μ chains.[5] On the other hand, we had one antiserum specific for rabbit IgG Fc that reacted well with plasma cells in fixed smears, but not at all with lymphocytes or plasma cells in suspension. The Fc determinants against which this antiserum was directed were presumably not available for reaction on the surface of lymphocytes.

The immunoglobulin molecules appear to be distributed on the surface of lymphocytes in discrete spots or patches in a way similar to that of various membrane components, like the histocompatibility antigens, the TL antigen, and others (15, 21, 22). In this respect our findings are different from those reported by Raff et al. (4) on normal mouse lymphocytes, and by Johansson and Klein (23) on human lymphocytes in cases of lymphoid leukemia; these authors have seen, by immunofluorescence, the immunoglobulins distributed as polar caps or half-moons. On the other hand, our observations are in agreement with those of Mandel et al. (24) who have seen that limited areas of the surface of a small number of mouse lymphocytes react with radioactive flagellin or haemocyanin.

It is unlikely that these differences are due to species differences in the distribution of the immunoglobulin molecules on the surface of lymphocytes. It would appear on the other hand that the caps may be the consequence of the alteration of lymphocyte surfaces due to prolonged treatment of cells in solutions of low protein content.[6]

It is clear from our results that 40–65% of lymphocytes in peripheral blood or lymphoid tissues in the rabbit do not have immunoglobulins that can be demonstrated with our experimental procedure. Apparently immunoglobulin-negative lymphocytes are also detected in comparable proportion in rabbit lymphocytes investigated with the mixed antiglobulin reaction (2), and amongst mouse lymphocytes studied either by immunofluorescence or with a very sensi-

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[5] Kelus, A. S., and B. Pernis. Manuscript in preparation.

[6] Pernis, B., and M. C. Raff. Unpublished observations.

tive method of immunoautoradiography (4). It is obviously impossible to con-
clude that these cells do not have surface immunoglobulins at all; these might,
in fact, be present in amounts inferior to the threshold of detection of the
methods. It might also be that in these cells the immunoglobulin molecules are
so connected with other membrane components that they are not available for
reaction with anti-immunoglobulin antisera.

It nevertheless appears, with regard to the surface immunoglobulins, that the
lymphocytes from different species fall into two categories, one in which the
surface reaction with anti-immunoglobulin antisera is readily demonstrable,
and the other for which this is not the case. There is some evidence (25) to sup-
port the possibility that the apparently negative cells may be thymus-derived
lymphocytes, and in fact all the thymus lymphocytes do not react with anti-
immunoglobulin antisera.

The discussion of the role of the immunoglobulins present on the surface of
lymphoid cells in the immunological functions of these cells can be very brief.
Obviously these immunoglobulins can react with antigens; this is borne out not
only by their apparent orientation with the Fab toward the outside, but also
by the direct demonstration of the specific binding of an antigen (horse ferritin)
in discrete spots on the surface of a small proportion of lymphocytes in non-
immunized rabbits, a proportion that increases considerably after immuniza-
tion and becomes impressive in the draining lymph nodes a few days after a
secondary stimulation with the antigen.

It follows that the immunoglobulins of the lymphocyte membrane fit within
the definition of antigen receptors and/or sessile antibodies, and that antibodies
of different specificity are present on the surface of different cells. The presence
on the surface of lymphoid cells of specific receptors for antigens has already
been shown with various techniques, such as those that rely on the adherence
of particulate antigens (bacteria or red cells) or on the binding of radioactive
antigen molecules (24, 26–29). In all of these experiments the immunoglobulin
nature of the receptors has been consistently supported by the inhibition of the
binding of the antigen with anti-immunoglobulin antisera.

It appears likely that a cell with immunoglobulin spots on its surface will
give a positive result when tested for binding of an antigen of corresponding
specificity; on the other hand, it remains to be investigated whether all the cells
that can be shown to bind antigen with one of the above mentioned techniques
have immunoglobulin spots that can be demonstrated with immunofluorescence.
Recent reports (30–32) would also indicate that thymus lymphocytes, which
do not have immunoglobulins demonstrable with immunofluorescence or auto-
radiography, can bind antigens in a specific way.

In any event, the immunofluorescent detection of immunoglobulins on the
membrane of lymphocytes provides a visual support of the concept of antigen
receptors on the surface of these cells, a concept that has been developed both

on the basis of experimental evidence (24, 26–29) and of theoretical considerations (33, 34).

SUMMARY

Small and medium lymphocytes from the peripheral blood and lymphoid tissues of the rabbit react in suspension with antibodies directed against different immunoglobulin determinants. Through immunofluorescence, it was possible to show that numerous discrete spots on the surface of the positive lymphocytes carry immunoglobulin molecules.

The positive lymphocytes are about one-half of all lymphocytes in the different preparations; thymus lymphocytes are all negative.

With antisera specific for rabbit IgM as well as with antisera directed against allotypic determinants specific for IgM or IgG, it was possible to show that about nine-tenths of the immunoglobulin-positive lymphocytes carry IgM molecules on their surface.

With antisera directed against a- and b-locus determinants, it was also possible to demonstrate that both heavy and light chains were present in the surface immunoglobulins. Furthermore, in animals which were heterozygous at the a or the b locus, it was found that each lymphocyte had immunoglobulins synthesized under the influence of only one of two alleles.

A very small proportion of lymphocytes could be shown to have a specific surface reaction with one antigen (horse ferritin); the proportion of these cells increased very much after immunization.

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