CELLULAR LOCALIZATION OF IMMUNOGLOBULINS WITH DIFFERENT ALLOTYPIC SPECIFICITIES IN RABBIT LYMPHOID TISSUES*

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Considerable attention has been devoted recently, to the genetically defined differences that may exist amongst the immunoglobulins of different individuals belonging to the same species. This phenomenon (allotypy), is not confined to the immunoglobulins, but it is particularly in this field that the presence of genetical markers allows a deeper insight in different physiological or pathological processes that are related with the synthesis of these molecules. One of the more interesting questions that, on the basis of the genetic markers, have been raised with regards to the synthesis of the immunoglobulins is whether, in an individual heterozygous at a given locus, the products of two allelic genes are manufactured by different immunoglobulin-producing cells.

Clear evidence for a differentiation of plasma cells with regards to the molecular classes (1, 2) or the antigenic type (3, 4) of the immunoglobulins that they produce has been obtained by immunofluorescence studies of human lymphoid tissues; when, however, this same method has been applied to the localization of gamma globulin allotypes in lymph node cells of heterozygous rabbits, then it was found (5) that the products (A4 and A5)[1] of two *allelic* genes were almost regularly present in the same cell.

As the results of Colberg and Dray (5) were complicated by the fact that the fluorochrome-conjugated antisera that had been used contained some of the antigenic determinants whose cellular localization was being studied, we decided to reinvestigate this problem with the use of antisera that did not react between themselves. In addition to the study of rabbits heterozygous at the *b* locus, we examined also animals heterozygous at the *a* locus and we have found

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[1] The notation for allotypy used throughout this article is that of Dray *et al.* (6).

that (with the exception of the elements of the germinal centers in which the localization of immunoglobulins by immunofluorescence gives a different pattern) the products of allelic genes are always present in different cells. This finding has been grounded on a more firm basis than that given by visual observations, with the use of fluorescence spectrography of single cells.

Materials and Methods

Animals.—A total of 15 adult rabbits have been studied. These animals may be divided in three groups according to the treatment to which they had been subjected before examination of the tissues. The animals of group A were immunized with twice crystallized horse ferritin. A total of 2 mg of this protein dissolved in saline were administered, 1 mg in each foot-pad; this treatment was repeated 5 times at weekly intervals. Seven days after the last of these injections in the foot-pads, the animals received 2 mg of ferritin intravenously; the intravenous treatment was repeated after a further 7 days and, at the same time, a booster injection of 1 mg was given in each foot-pad. The animals were sacrificed 4 days afterwards. The animals of group B received in each foot-pad a mixture of 0.2 ml of whole human serum and 50 μg of formol-killed *Escherichia coli*; this treatment was repeated 4 times at weekly intervals and the animals were sacrificed 4 days after the last injection. The animals of group C did not receive any immunizing treatment.

The rabbits of groups A and B were obtained from closed colonies and were the progeny of parents with known allotypic specificities; the rabbits of group C were randomly bred, commercially available animals. The allotypic specificities were determined by micro-Ouchterlony diffusion with reference antisera. The allotypic specificities found in each rabbit are summarized in Table I.

Antisera.—Antisera reacting with different allotypes were prepared with the method of Dubiski *et al.* (7). The recipient rabbits lacked only one of the allotypic specificities present in the gamma globulin of the donor rabbit and the antisera were therefore specific reactants for that allotype; the specificity of the antisera was tested by micro-Ouchterlony gel precipitation with different rabbit sera. The antigenic as well as the antibody specificities of the rabbit antisera used in the present work are listed in Table II.

The antiserum against rabbit gamma globulin has been prepared by repeated immunizations of a goat with purified alcohol-fractionated (Fraction II) rabbit gamma globulin. When tested in immunoelectrophoresis against whole rabbit serum it gave a long and strong precipitin line against rabbit gamma G globulin, and a faint arc in the beta region. After absorption with small amounts of L chains (prepared from rabbit gamma globulins with the method of Fleischman *et al.*, (8) this antiserum no longer reacted with isolated rabbit macroglobulin (prepared from whole rabbit serum with the method of Reisner and Franklin, (9) and was then considered to be a specific anti-rabbit gamma G antiserum.

The anti-horse ferritin antiserum was obtained from the rabbits of group A at the moment of sacrifice.

Preparation of Conjugates.—The globulin fraction of each antiserum described in the preceding section was prepared by precipitation with ammonium sulfate. The antisera were diluted with the addition of an equal volume of distilled water and cooled at 5°C. To the diluted antiserum an equal volume of cold saturated ammonium sulfate solution was then added dropwise. The resulting precipitate was washed three times with half-saturated ammonium sulfate solution and then dissolved in saline in a volume equivalent to one-half or one-third of the original serum. The residual ammonium sulfate was finally removed by dialysis, in the cold, against normal saline until the dialysis fluid was free of SO_4 ions (tested by absence of precipitation after addition of $BaCl_2$).

These globulin fractions were conjugated with fluorescein isothiocyanate (Sylvana Co., Millburn, New Jersey), or with tetramethylrhodamine isothiocyanate (Baltimore Biochemical Corporation, Baltimore), with the method of Coons (10), employing 0.05 mg of the fluorochrome per mg of the protein; the particular fluorochrome used for a given antiserum is indicated in Table II. After conjugation the free fluorochrome was separated from the conjugate by passage through a column of sephadex G-25 medium (Pharmacia, Upsala, Sweden); the conjugates were tested for antibody activity against different rabbit sera by agar gel precipitation and then divided into small aliquots which were preserved at −20°C. Immediately before use the conjugates were invariably absorbed twice with an equal amount of guinea pig liver powder as indicated by Coons (10).

TABLE I

Allotypic Specificities in Rabbits Used as Tissue Donors for Immunofluorescence Studies of Spleens and Lymph Nodes

Rabbits	Allotypic specificities present	
	a locus	*b* locus
Group A		
RB 5530	A1	A4
RB 5561	A1	A6
RB 5379	A2	A4
RB 5557	A1, A2	A4
RB 5247	A1	A4, A6
RB 5549	A1, A2	A4, A6
RB 5560	A1, A2	A4, A6
Group B		
RB 5601	A1	A4
RB 5663	A1	A6
RB 5589	A1	A4, A6
RB 5600	A1, A2	A4, A6
Group C		
V 22	A2, A3	A4, A5
CL 262	A1	A4, A5
CL 263	A1, A2	A4
CL 264	A1, A3	A4

Preparation of Tissue Sections.—Popliteal lymph nodes and spleens were collected from rabbits of group A; in addition to these tissues mesenteric lymph nodes were also collected from animals of groups B and C. Small tissue blocks were cut and, after blotting quickly, wrapped in aluminum foil and then immersed for at least 2 minutes in isopentane cooled at −70°C with dry ice; the frozen tissues were then kept at −20°C for periods not exceeding 1 month. The tissues were sectioned in a Linde cryostat at −20°C with a Jung microtome, the thickness of the sections was 4 to 5 μ. The sections were transferred on thin glass slides (or on quartz coverslips when necessary for fluorescence spectroscopy), quickly thawed by applying a finger on the reverse side and then dried under reduced pressure at room temperature for about 10 minutes.

Fixation and Staining of Tissue Sections.—Adjacent sections from the various tissues were subjected to the following fixation and staining procedures:

1. Fixation in absolute ethyl alcohol for 10 minutes and staining with methyl green–pyronin. This gave a general picture of the distribution of plasma cells and other pyroninophilic cells in the tissues.

2. Fixation in cold acetone at room temperature for 10 minutes, followed by drying under reduced pressure and then by application of the conjugated antisera. This was done with the method of Coons and Kaplan (11); for the demonstration of anti-horse ferritin antibodies the "sandwich" technique of Coons *et al.* (12) was used. On a number of sections sequential staining for two different allotypic determinants (or for one allotypic determinant and rabbit gamma globulin) was performed. In this case the section was flooded with one conjugate for 30 minutes, the excess conjugate was washed off with phosphate-buffered (pH 7.2) saline by letting a considerable amount (about 20 ml) of this solution to flow dropwise onto the section;

TABLE II

Rabbit antisera used for the immunofluorescence studies

Antiserum	Antigenic specificity		Antibody specificity	Fluorochrome
	a locus	*b* locus		
5146/III	A3	A5	Anti-A1	Rhodamine
6719/II	A3	A4	Anti-A1	Rhodamine
5279/III	A3	A5	Anti-A2	Fluorescein
5153/II	A3	A5	Anti-A4	Rhodamine
5153/II	A3	A5	Anti-A4	Fluorescein
5429/II	A1	A6	Anti-A4	Rhodamine
5183/II	A1	A4, A6	Anti-A5	Fluorescein
5253/II	A2, A3	A5	Anti-A6	Fluorescein

after sucking off the excess moisture with filter paper applied to one edge of the section, the second antiserum (conjugated with a different fluorochrome) was applied. In all instances in which two different antisera were used on the same section, the absence of interreactivity of the two antisera used was checked by placing these same antisera in adjacent wells in a micro-Ouchterlony plate; as a consequence of this absence of interreaction it was found that, in general, the same results were obtained with a given pair of antisera, regardless of which conjugate was used first. An exception to this rule was the staining for one allotype and for total gamma globulin; here it was thought advisable to use the antiallotype conjugate first and the anti–gamma globulin second, to avoid interference with the binding of the anti-allotype antibodies on sites located near those occupied by the previously fixed anti–gamma globulin antibodies.

In addition a given sequence had to be used when staining the same section for the A4 and A5 allotypes; in this case, due to the difficulty of preparing a specific anti-A5 antiserum in a rabbit of genotype A^{6}_{b}, A^{6}_{b}, completely non-interreacting antisera were not available and we had to use an anti-A5 antiserum prepared in an A^{4}_{b}, A^{6}_{b} rabbit (see Table II). The staining was then performed with the anti-A4 first followed by the anti-A5; this procedure avoided the formation of a "sandwich" due to the second antiserum reacting with *antigenic* determinants of the first, but did not prevent the possibility that unsaturated antibody sites of the first antiserum might react with *antigenic* determinants of the second.

To ascertain the specificity of the various immunofluorescence stainings, all the customary controls (such as inhibition by prior treatment with unconjugated antiserum etc.) were performed; however, in the case of double staining and when these evidenced different antigenic determinants in different cells, a sort of built-in control was given by the demonstration of groups of plasma cells in which alternate elements fluorescing with different colors were visible (see for instance Fig. 6). This in fact could be hardly due to any sort of artifact.

Fluorescence Microscopy.—A Leitz ortholux microscope was used equipped with a HBO 200 ultraviolet source. The light was filtered with UG1 (1.5 mm) and BG38 filters; generally a cardioid immersion type darkfield condenser was used. Photographs were taken on ektachrome high speed 23DIN daylight type 35 mm film. In our hands this resulted in pictures in which the fluorescence of rhodamine appeared slightly more yellow and that of fluorescein slightly more blue than in pictures taken with superanscochrome; the latter film was, however, no longer available.

Television Microspectrography of Fluorescence of Single Cells.—[2] The sections were stained on the same day or the day before the observations and were flown to Wetzlar in a vacuum desiccator kept at 2°C in an ice bag. The instrumentation used for fluorescence spectroscopy was similar to that used by Loeser and West (13) to study the fluorescence spectrum of the nucleus of single cells stained with acridine dyes.

Briefly it consisted of a Leitz microspectrograph equipped with an Osram HBO 200 mercury arc lamp, primary filter UG1 (1.5 mm) and BG38 and ultraviolet abs. secondary filters, immersion dark field condenser and × 300 reflecting objective. As a result of the 300 magnification of the object image in the plane of the slit, a measuring field of only 1 μ is obtained in the object plane with a slit width of as little as 0.3 mm. The dissector tube of an orthicon television camera replaced the spectrograph plate: it transmits the object spectrum on the screen of the television receiver (monitor) in the form of amplified video signals. A line selector switch permits the reproduction of the video signal of any line of the television image on the screen of an oscilloscope. The tracings that are thus recorded give a measure of the intensity of the light as a function of its wavelength.

The calibration of wavelengths was made on the basis of the reference emission spectrum of a high pressure mercury lamp, which had the main peaks of the emission curve at 578, 546, 436, 405, and 364 mμ.

Fluorescence spectra of single cells were taken by bringing the image of the cytoplasm in correspondence with the entrance slit of the spectrograph and by recording the oscilloscope tracing; this was a measure of the emission spectrum of the fluorochrome or fluorochromes bound by the conjugated antibodies to the cytoplasm of a single plasma cell. Inasmuch as, for the purposes of the present work, we were only interested in differentiating the fluorescence spectra of rhodamine and fluorescein, no attempts were made to correct the emission spectra for the spectral response of the television camera tube and the secondary filters, nor for the non-linearity of response of the camera tube itself; the raw data were instead used, and the Text-figs. 1 to 4 represent directly the oscilloscope tracings. The position of the image on the oscilloscope grid may vary a little in recordings taken at different times, and therefore to obtain precise informations on the wavelength of the peaks of emission spectra it would be necessary to take alternate recordings of the experimental object and of the reference lamp. This was however unnecessary for the purposes of the present investigation, which were only those of identifying the presence or absence of mixtures of the two fluorochromes.

This method is capable of yielding objective and semiquantitative information on the

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[2] The spectrographic observations were performed in the research laboratories of the Ernst Leitz GmbH in Wetzlar, Western Germany. The authors wish to express their gratitude to the Ernst Leitz GmbH, that made the instrumentation available, and to Dr. A. Thaer whose assistance made the recording possible.

fluorochrome bound to the cytoplasm of a single plasma cell in sections of tissues subjected to double immunofluorescence staining. In order to obtain an estimate of the sensitivity of the method, that is of how much rhodamine might be detected if admixed with fluorescein in the cytoplasm of a single cell (and *vice versa*), sections from a rabbit spleen were stained with various mixtures of an anti-gamma G antiserum conjugated with fluorescein or with rhodamine isothiocyanates. It was seen that, under our experimental conditions, a definite modification of the outline of the fluorescence spectrum from that of pure fluorochrome could still be detected from cells in sections stained with a mixture of 95 per cent fluorescein-conjugated and 5 per cent rhodamine-conjugated anti-gamma globulin antibodies and *vice versa*. The sensitivity of the spectrographic method in detecting mixtures of fluorochromes thus surpasses that of simple visual observation, since in the same experiment cells with 80 per cent fluorescein and 20 per cent rhodamine were already classified as "green" by visual observation alone. Further details on the quantitative aspects of television microspectrography applied to immunofluorescence of single cells will be published elsewhere.

RESULTS

General considerations and observations.—The animals of group A showed, without exception, a good antibody response to horse ferritin at the time of sacrifice; they had precipitating antibodies in the serum in amounts variable from 0.7 to 2.3 mg antibody protein/ml, and their spleens and popliteal lymph nodes showed histological reactions correlated with the antibody response. These were more marked in the popliteal lymph nodes in which the methyl green–pyronin stain revealed a high number of plasmablasts and plasma cells in the medullary cords; numerous secondary follicles were present in the cortex. When adjacent sections were stained with fluorescein-conjugated anti-gamma G globulin antiserum, a good correspondence was found between the pyroninophilic cells in the medullary cords and the elements containing variable amounts of gamma globulins; the majority of the germinal centers in the secondary follicles were also positive for gamma globulins giving the well known reticulate pattern of immunofluorescence (14). With the staining for anti-horse ferritin antibodies many of the plasmablasts and plasma cells in the medullary cords also appeared positive; anti-horse ferritin antibodies were also present in the germinal centers but their distribution did not appear to be completely superposable to that of gamma globulins. In the spleens of the animals of group A the changes related to the production of antibodies were less prominent than in the lymph nodes, however many plasmablasts and plasma cells that could be stained for gamma globulin or anti-ferritin antibody were present in the red pulp and in the periarteriolar sheaths (Fig. 1); gamma globulin–containing germinal centers were also present.

In the spleens and popliteal lymph nodes of the animals of group B the overall picture obtained with the methyl green–pyronin and anti-gamma globulin stainings was similar to that of the rabbits of group A; no staining for specific antibodies was attempted in this second group of animals.

In group C the number and disposition of gamma globulin–containing cells was rather different from one animal to another, presumably reflecting

differences in the degree or nature of "spontaneous" antigenic stimulation that had occurred; it must be stressed, however, that the animals of group C listed in Table I were those that, out of a larger number of rabbits, were selected as having a large number of plasma cells and their precursors in the spleens; here these elements were present in the red pulp and, even more numerous, at the level of the periarteriolar sheaths.

For a preliminary test of the ability of conjugated antiallotype antisera to stain the gamma globulin–containing cells of rabbit spleens and lymph nodes, the tissues of rabbit RB 5530 (genotype A_a^1 A_a^1, A_b^4 A_b^4) were stained with a fluorescein- or a rhodamine-conjugated anti-A4 antiserum. Both conjugates gave beautiful staining of plasmablasts, plasma cells, and germinal centers; no apparent differences were observed in the intensity of staining by the anti-allotype and by the anti–gamma globulin anti-sera; also the microscopic details at the cellular level were the same so that a section stained by a conjugated antiallotype was practically not distinguishable from the companion one stained with the anti–gamma globulin antiserum (see, however, the experiments of double staining for the presence of some cells that reacted with the anti–gamma globulin antiserum only).

Results of Double Staining for Two Different Allotypes.—

Rabbits homozygous at both loci: Two animals of genotype A_a^1 A_a^1, A_b^4 A_b^4 (RB 5530 and RB 5601) were examined. In each case sections from the spleens and popliteal lymph nodes were stained first with rhodamine-conjugated anti-A1 antiserum followed by fluorescein-conjugated anti-A4. The majority of the elements of the plasma cell series were stained by both conjugates and showed a yellow fluorescence (Fig. 2); some cells, however, were seen that reacted only with anti-A1 or with anti-A4 (see Table III); similar findings were obtained when the tissues were stained first with the anti-A4 antiserum and then with the anti-A1. The germinal centers of the lymphoid follicles were always stained by both antisera. Practically the same results were obtained with the tissues of the 2 rabbits of genotype A_a^1 A_a^1, A_b^6 A_b^6 (RB 5561 and RB 5663) stained with rhodamine-conjugated anti-A1 followed by fluorescein-conjugated anti-A6; even in these animals the majority of the cells that reacted with anti-A1 also reacted with anti-A6; there were, however, some elements positive only with one or the other of the two conjugates.

Rabbits heterozygous at one locus: Rabbits heterozygous at one locus have, obviously, three different allotypes that can be stained. Lymphoid tissues from three rabbits heterozygous at the *b* locus (RB 5247 and RB 5589 of genotype A_a^1 A_a^1, A_b^4 A_b^6, and CL 262 of genotype A_a^1 A_a^1, A_b^4 A_b^5) have been investigated with double staining for two different allotypes. The main object of this investigation was the cellular localization of the products of the two allelic genes at the *b* locus. This could be easily done for the animals RB 5247 and RB 5589,

TABLE III

Results of Double Staining for Two Allotypes or for One Allotype and Gamma G Globulins on Cells of the Plasma Cell Line in the Spleen (Excluding Germinal Centers) of Different Homozygous or Heterozygous Rabbits (Counts on 600 Cells)

Rabbit	Rabbit genotype	Antisera	Stained cells		
			per cent	*per cent*	*per cent*
RB 5530	$A_a^1\ A_a^1,\ A_b^4\ A_b^4$	Anti-A1 + anti-A4	(A1 + A4) 80	(A1) 10	(A4) 10
RB 5601	$A_a^1\ A_a^1,\ A_b^4\ A_b^4$	Anti-A1 + anti-gamma G	(A1 + γG) 69	(A1) 0	(γG) 31
RB 5589	$A_a^1\ A_a^1,\ A_b^4\ A_b^6$	Anti-A4 + anti-A6	(A4 + A6) 0	(A4) 50	(A6) 50
		Anti-A1 + anti-A4	(A1 + A4) 47	(A1) 36	(A4) 17
		Anti-A1 + anti-A6	(A1 + A6) 43	(A1) 44	(A6) 13
RB 5247	$A_a^1\ A_a^1,\ A_b^4\ A_b^6$	Anti-A4 + anti-A6	(A4 + A6) 0	(A4) 45	(A6) 55
		Anti-A1 + anti-A4	(A1 + A4) 43	(A1) 39	(A4) 18
		Anti-A1 + anti-A6	(A1 + A6) 41	(A1) 41	(A6) 18
V 22	$A_a^2\ A_a^3,\ A_b^4\ A_b^5$	Anti-A4 + anti-A5	(A4 + A5) 0	(A4) 53	(A5) 47
RB 5600	$A_a^1\ A_a^2,\ A_b^4\ A_b^6$	Anti-A1 + anti-A2	(A1 + A2) 0	(A1) 62	(A2) 38
CL 262	$A_a^1\ A_a^1,\ A_b^4\ A_b^5$	Anti-A4 + anti-A5	(A4 + A5) 0	(A4) 61	(A5) 39
		Anti-A1 + anti-gamma G	(A1 + γG) 88	(A1) 0	(γG) 12
CL 263	$A_a^1\ A_a^2,\ A_b^4\ A_b^4$	Anti-A4 + anti-gamma G	(A4 + γG) 86	(A4) 0	(γG) 14
CL 264	$A_a^1\ A_a^3,\ A_b^4\ A_b^4$	Anti-A4 + anti-gamma G	(A4 + γG) 85	(A4) 0	(γG) 15

by staining sections of spleens and popliteal lymph nodes with rhodamine-conjugated anti-A4 (antiserum 5153/II) followed by fluorescein-conjugated anti-A6 (antiserum 5253/II); these two antisera did not interreact one with another when tested in micro-Ouchterlony plates and, consequently, did not do so in tissue sections. The results were clear cut in both animals, and it could be shown that the elements of the plasma cell series in the spleen and in lymph nodes were invariably positive with one *or* with the other of the two antisera (Fig. 3). Counts made on 600 elements in the spleens of each animal showed that the cells containing the A4 allotype were about as numerous as those containing the A6 allotype (Table III). In general the two types of cells were mixed at random, but in some areas in the lymph nodes a preponderance of one or the other was observed; no differences were remarked concerning the cytological appearance of the two types. This separation of the two allotypes dependent on the A_b^4 and A_b^6 alleles was the rule with the immunoglobulin-containing cells found in lymphoid tissues outside of the germinal centers of the secondary follicles, irrespective of whether a given cell had the cytological appearance of a plasmablast or of a more or less mature plasma cell. In contrast the double staining did not differentiate two populations of cells amongst the elements of the germinal centers; in fact those centers that contained immunoglobulins in the lymphoid follicles of the spleen or lymph nodes, could always be uniformly stained by both antisera and no areas in the network of immunofluorescence that characterized the centers appeared to take up preferentially one of the antibodies rather than the other (Fig. 4). In addition similar patterns were seen when the same center was localized in adjacent sections alternatively stained with one antiserum only.

In the 3rd rabbit heterozygous at the *b* locus (genotype $A_a^1\ A_a^1,\ A_b^4\ A_b^5$) the double staining for the A4 and A5 allotypes, met with the difficulty that one of the two fluorescent antibodies (the anti-A5 prepared in a rabbit of genotype $A_a^1\ A_a^1,\ A_b^4\ A_b^6$) bore antigenic determinants with which the other antiserum (the anti-A4, prepared in a rabbit of genotype $A_a^1\ A_a^1,\ A_b^6\ A_b^6$) was capable of reacting. This unfavourable condition could be partially overcome by staining first with the (rhodamine-conjugated) anti-A4 followed by the (fluorescein-conjugated) anti-A5; with this procedure the findings (Fig. 5) in the rabbit of genotype $A_a^1\ A_a^1,\ A_b^4\ A_b^5$ were basically similar to those in the rabbit of genotype $A_a^1\ A_a^1,\ A_b^4\ A_b^6$, in that the staining for the products of two allelic genes at the *b* locus revealed two populations of plasma cells. In spite of the fact that the differences in the fluorescence colors between the two classes of plasma cells were not as clear-cut as in the case of double staining for A4 and A6 (see the results of microspectrography), a count could be done on 600 of these elements in the spleen and it was found that the cells containing immunoglobulins with the A4 allotype were more numerous (61 per cent) than those containing the A5 allotype

(39 per cent). Again in the elements of the germinal centers no differentiation was found.

Rabbits heterozygous at both loci: Three doubly heterozygous rabbits were examined: RB 5600 and RB 5549 (of genotype $A_a^1 A_a^2$, $A_b^4 A_b^6$), and V 22 (genotype $A_a^2 A_a^3$, $A_b^4 A_b^5$).

In these animals the double staining for the two allotypes at the *b* locus gave the same results as in rabbits heterozygous at the *b* locus only; here again a complete separation was found, with cells containing A4 *or* A6 (in RB 5600 and RB 5549) (Fig. 6) and, respectively A4 *or* A5 (in V 22); in the spleen of V 22 600 cells were counted and 53 per cent of them appeared to contain the A4 allotype and 47 per cent the A5. Again a mixed staining was seen in the germinal centers.

Double staining for two allotypes at the *a* locus was performed on the spleens and lymph nodes of RB 5600 and 5549, utilizing a rhodamine-conjugated anti-A1 (antiserum 5146/III) and a fluorescein-conjugated anti-A2 (antiserum 5279/III); this staining revealed cells positive for A1 and cells positive for A2 (Fig. 7); mixed staining was limited to the elements of the germinal centers. Amongst 600 cells of the plasma cell series in the spleen of RB 5600, 62 per cent reacted with the anti-A1 and 38 per cent with the anti-A2 antiserum.

Finally the lymphoid tissues of RB 5600 and RB 5549 were subjected to double staining for one allotype at the *a* locus and one allotype at the *b* locus. The following combinations were tested: anti-A1 and anti-A4, anti-A1 and anti-A6, anti-A2 and anti-A4, anti-A2 and anti-A6. As was expected this kind of double staining revealed cells that reacted with both antisera and cells that reacted with one antiserum only; the number of cells that showed a mixed staining was, in general, around one-third of all the elements showing immunofluorescence of any kind; precise counts were not made, however.

Therefore, to summarize the results of double staining for two different allotypes, it can be said that the staining for the products of two allelic genes in heterozygous rabbits always showed that these were found in different cells; the only notable exception to this rule was given by the cells of the germinal centers. On the other hand, when staining for two allotypes determined by different loci, many cells containing both allotypes were seen.

Results of Double Staining for One Allotype and Gamma G Globulins.—Since it has been shown (15, 16) that not all the gamma G globulin molecules present in the serum of a given rabbit carry the known allotypic determinants, it was of interest to investigate the possibility that separate cells might produce these apparently "allotype-lacking" molecules. The simplest way to do this, by immunofluorescence, was that of staining the lymphoid tissues of rabbits homozygous at at least one locus, with an antiserum directed against the allotype determined by that locus and with a specific heterologous anti-gamma G antiserum, conjugated with different fluorochromes.

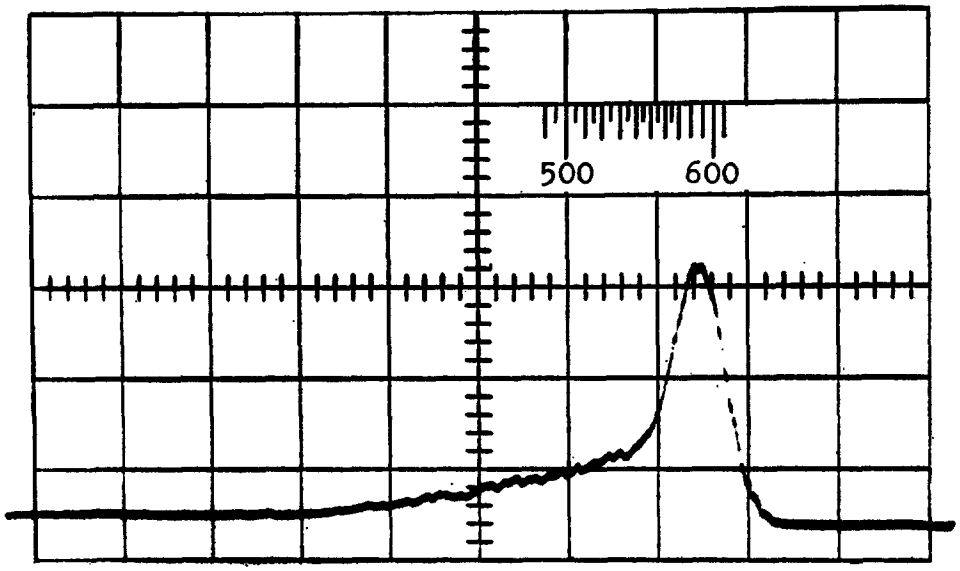
This has been done on the tissues of RB 5601 (genotype A_a^1 A_a^1, A_b^4 A_b^4) and of CL 262 (genotype A_a^1 A_a^1, A_b^4 A_b^5), by applying first a rhodamine-conjugated anti-A1 antiserum followed by a fluorescein-conjugated anti-rabbit gamma G antiserum. In the spleens and lymph nodes of both rabbits the majority of the cells of the plasma cell series took up both antisera and appeared yellow; there was, however, a sizeable number of cells that reacted with the anti-gamma G antiserum only. The number of these apparently "*a*-negative" cells was different in the two rabbits; counts made on 600 elements in the spleen of RB 5601 gave 31 per cent of the cells not reacting with the anti-A1 antiserum (5146/III), whereas amongst 600 elements of the plasma cell line in the spleen of CL 262 only 12 per cent reacted with the antigamma G and not with the anti-A1 (antiserum 6719/II). We did not see cells that were positive with the antiallotype only; this was expected because the antiallotype being itself a gamma G, it determined the secondary fixation of the anti-gamma G antibodies on every cell on which it was bound, even if the cytoplasm originally contained gamma A or gamma M globulins.

The lymphoid tissues of 2 rabbits homozygous at the *b* locus (CL 263 of genotype A_a^1 A_a^2, A_b^4 A_b^4 and CL 264 of genotype A_a^1 A_a^3, A_b^4 A_b^4) were subjected to double staining for the detection of cells containing gamma G globulins lacking the A4 allotype; this was done by first staining with rhodamine-conjugated anti-A4 antiserum (5429/II in both cases) followed by fluorescein-conjugated anti-gamma G. Here again the majority of the cells reacted with both antisera, but cells that reacted with the anti-gamma G and not with the anti-A4 antiserum, and therefore showed a pure green fluorescence (Fig. 8) were easily identified; their relative number was 14 per cent in the spleen of CL 263 and 15 per cent in the spleen of CL 264.

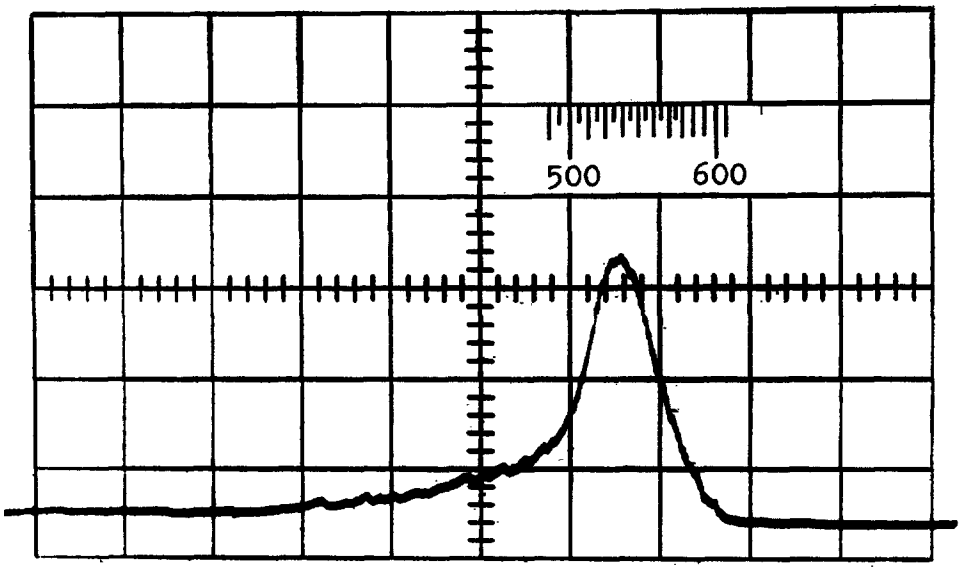
Results of Television Microspectrography of Fluorescence of Single Cells.—This method was applied to sections of lymphoid tissues from rabbits heterozygous at the *b* locus and subjected to double staining for the A4 and A6 allotypes (RB 5247 and RB 5589); the spectra (Text-figs. 1 and 2) of more than 25 cells from each animal always showed that a given cell of the plasma cell series (outside of the germinal centers) was stained by fluorescein *or* by rhodamine. In consequence of what has been established on the sensitivity of the microspectrographic method in detecting mixtures of two fluorochromes, it can be assessed that in A_b^4 A_b^6 heterozygotes a single cell of the plasma cell line contains gamma globulin molecules 95 to 100 per cent of which are marked by the A4 *or* by the A6 allotypes.

The results of the double staining for two different allotypes determined by allelic genes at the *a* locus were also checked by microspectrography and the spectra obtained from the tissues of RB 5600 and RB 5549 confirmed the visual observation that localized the A1 and A2 allotypes in different cells.

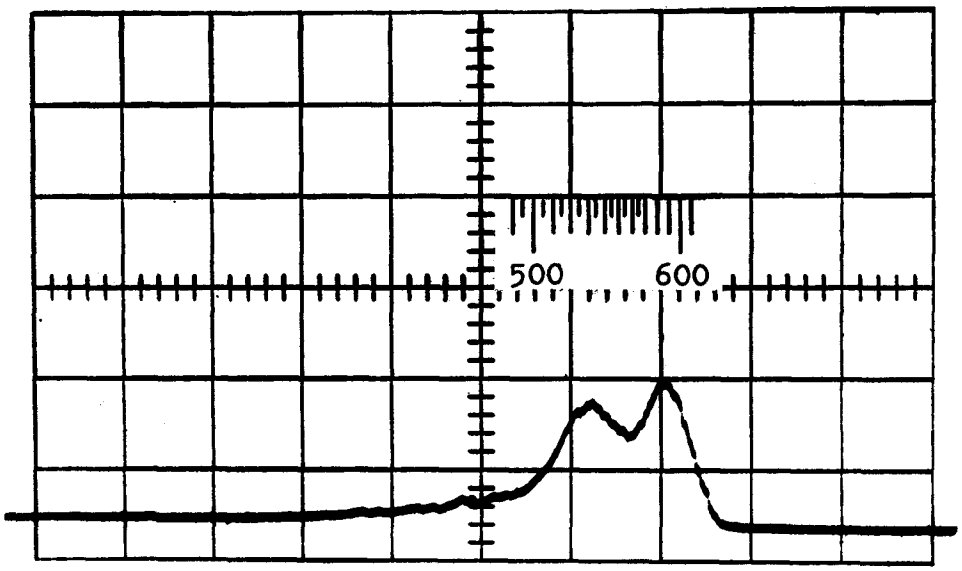
Instead pure fluorescence emission spectra of fluorescein or rhodamine were



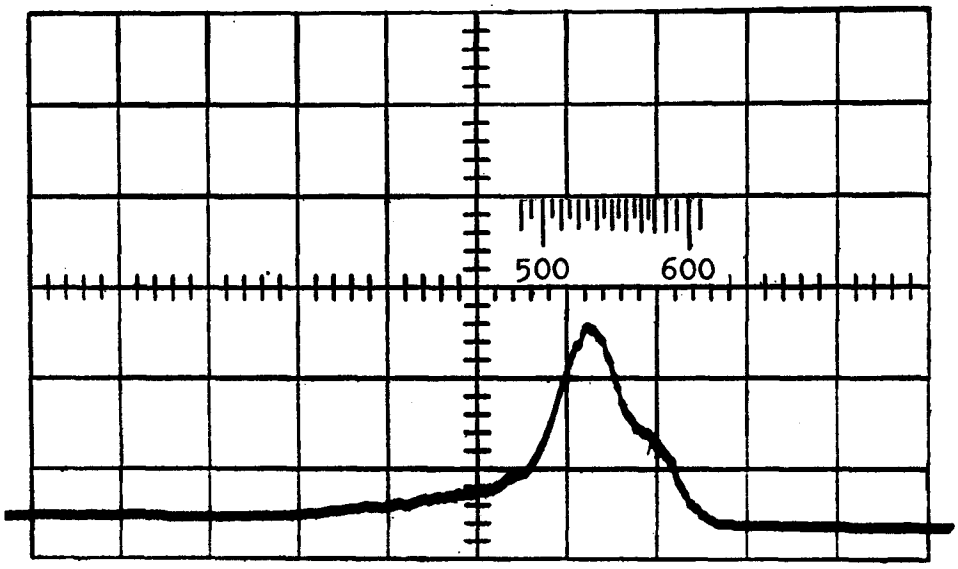
TEXT-FIG. 1. Same spleen and staining as in Fig. 3. Fluorescence emission spectrum from a single cell stained by the anti-A4 antiserum: the oscillographic tracing shows the pure spectrum of rhodamine with maximum at 590 mμ.



TEXT-FIG. 2. Same spleen section as Text-fig. 1. Fluorescence emission spectrum from a single cell stained by the anti-A6 antiserum: the tracing shows the pure spectrum of fluorescein with maximum at 525 mμ.



TEXT-FIG. 3. Same spleen and staining as in Fig. 5. Fluorescence emission spectrum from a single cell stained predominantly by the rhodamine-conjugated anti-A4 antiserum; the binding of considerable amount of anti-A5 also is shown by the second peak with maximum at 525 mμ.



TEXT-FIG. 4. Same spleen section as Text-fig. 3. Fluorescence emission spectrum from a single cell stained predominantly by the fluorescein-conjugated anti-A5 antiserum; the binding of minor amounts of rhodamine-conjugated anti-A4 antibodies is shown by the modification of the outline of the tracing around 590 mμ, with respect to the spectrum of pure fluorescein.

not obtained from single cells of rabbits heterozygous at the *b* locus after double staining with conjugated antisera reacting with the A4 and A5 allotypes; in this case an appreciable amount of fluorescein-conjugated anti-A5 antibodies appeared to be fixed on the cytoplasm of cells containing the A4 allotype (Text-fig. 3); on the other hand a smaller amount of rhodamine-conjugated anti-A4 antibodies was demonstrable by microspectrography on the cytoplasm of the cells that contained the A5 allotype (Text-fig. 4). While the presence of some fluorescein on the cells predominantly marked by the rhodamine-conjugated anti-A4 antibodies, could be guessed by visual observation, the amount of rhodamine-conjugated anti-A4 antibodies on the cytoplasm of cells marked by the fluorescein-conjugated anti-A5 was too small for visual detection.

The presence of some degree of mixed staining in the cells of rabbits whose genotype at the *b* locus was A^4_b A^5_b, raises the possibility that, in this particular case, the allotypic determinants coded by allelic genes, may indeed be present in the same cell, although in very unequal amounts in two different groups of cells. We must point out, however, to the possibility of artifacts arising as a consequence of the presence in the antibody molecules of one antiserum (the anti-A5) of antigenic determinants with which the other antiserum could react.

The appreciable amount of (fluorescein-conjugated) anti-A5 bound by the cells that had reacted with the anti-A4 antibodies might have been due to the fact that not all the combining sites of the anti-A4 antibodies were saturated by the corresponding antigenic determinants in the cells, and therefore could bind additional antigen when this was presented, in the second stage of the staining, in the form of anti-A5 antibodies. Furthermore, during the second stage of the staining, a small amount of anti-A4 antibodies previously bound to the cytoplasm of the A4-positive cells, might be dissolved in antigen excess, due to the presence of A4 determinants in the anti-A5 antibody molecules and these complexes (that have free anti-A5 antibody-combining sites) might conceivably carry some rhodamine-conjugated anti-A4 molecules to the cytoplasm of the anti–A5-positive cells; alternatively a little anti-A4 antibody might have been left diffusely and loosely adsorbed to various structures in the sections after the first washing and might subsequently have been carried to the cytoplasm of the A5-positive cells through a similar mechanism. Experiments are now under way to define the possible role of these different artifacts.

Finally microspectrography was applied to the study of single cells of rabbits of genotype A^1_a A^1_a, A^4_b A^4_b (RB 5530 and RB 5601), stained with fluorescein-conjugated anti-A4 and rhodamine-conjugated anti-A1. As already mentioned the majority of the cells, in this experiment, had apparently reacted with both antisera and appeared yellow; this was confirmed by microspectrography.

DISCUSSION

The Cellular Localization of Allotypes Determined by Allelic Genes.—Our immunofluorescence observations indicate that single cells of the plasma cell series

that are present in the lymphoid tissues of heterozygous rabbits contain gamma globulins that carry one or the other of the allotypic markers determined by genes at the same locus, the only apparent exception being that of the elements of the germinal centers in secondary lymphoid follicles. This finding, that implies a selective inactivation of one of the two allelic genes in a given immunoglobulin-producing cell, is not in accord with the observations of Colberg and Dray (5) who have stained with anti-A4 and anti-A5 antisera, conjugated with different fluorochromes, the mesenteric lymph nodes of A_b^4 A_b^5 heterozygotes and have found a mixed staining not only of the elements of the germinal centers but also of 99 per cent at least of the cells of the plasma cell line in the medulla. The reasons of this discrepancy are not readily apparent; it is unlikely that it may be due to differences in the tissues examined or in their immunological stimulation because, although we have made most of our observations and cell counts on the spleens and popliteal lymph nodes of hyperimmunized rabbits, we obtained similar findings on the mesenteric nodes of 2 animals that had not been experimentally immunized (V 22 and CL 262) and therefore on material similar to that on which Colberg and Dray performed most of their observations. On the other hand our difficulties with the double staining for two allotypes by means of two antisera one of which carried antigenic determinants with which the other could react (as in the case of double staining for A4 and A5 allotypes) emphasize the possibility of technical artifacts in double staining with interreacting antisera; it is possible that these artifacts may completely obscure the selective detection of the two allotypes in different plasma cells when not only one but both the antisera used in the double staining are capable of mutual interreaction. These artifacts could not, obviously, have happened in the staining of consecutive sections with the two different antisera, a technique also employed by Colberg and Dray in an effort to localize the A4 and A5 allotypes in the same or different structures in the lymphoid tissues of heterozygous rabbits; with this method, however, it is very difficult to study a single cell, and groups of cells or germinal centers are expected to react with both antisera because of the random distribution of cells positive for one or the other of the two allotypes in the plasma cell clusters (Fig. 6), and because mixtures of immunoglobulins carrying different allotypes seem in fact to be present in a single center (Fig. 4). Finally it should be noted that some evidence for mixtures of the A4 and A5 allotypes in the cytoplasm of a single plasma cell was obtained by Colberg and Dray in restaining experiments in which the second antiserum was applied to the same sections after quenching the fluorescence of the first by prolonged exposure to ultraviolet light; we did not test this technique and we have no explanation as to why the application of the second antiallotype antiserum in these experiments failed to increase more than a few per cent of the number of fluorescent plasma cells seen at the first stage of the staining.

The next point to be discussed is that of the relationship between the per-

centage of cells marked by a given allotype in the spleen of heterozygous rabbits (Table III) and the relative amount of gamma globulin molecules with that allotype present in the serum. We did not determine this relative amount directly in the serum of the rabbits whose tissues we examined by immunofluorescence, therefore we can only compare the results of our cell counts with the data from the literature that report the percentages of molecules marked by the different allotypes in the sera of rabbits whose genotype, at the *a* or at the *b* locus, was the same as that of our animals. These data are those of Dray and Nisonoff (17), who in two $A_b^4 A_b^5$ heterozygotes, found that 64 to 66 per cent of the gamma G molecules reacted with an anti-A4 antiserum, while only 26 to 24 per cent were precipitated by an anti-A5; our observations on two similar heterozygotes (Table III) likewise revealed that cells with gamma globulins carrying the A4 allotype were more numerous than cells with gamma globulins carrying the A5, but their prevalence was less than expected on the basis of the above mentioned serological observations. It is possible that the ratio of A4 to A5 molecules may vary somewhat in individual $A_b^4 A_b^5$ heterozygotes since Leskowitz (18) found in one such rabbit an A4:A5 ratio of 63:36, a ratio which is very close to that of 62:38 found by Bornstein and Oudin (19) as the mean value amongst several $A_b^4 A_b^5$ heterozygotes. It is interesting to point out that this last ratio is practically the same as that (61:39) found by us for A4 to A5 plasma cells in the spleen of rabbit CL 262.

It appears therefore likely that, in heterozygous rabbits, the relative amounts of serum gamma globulins that carry one of the two allotypic determinants, is determined by the relative number of plasma cells that produce them rather than by the ratio of the synthesis of the two allotypes in the cytoplasm of a given cell.

Several facts that have been observed in the study of gamma globulin allotypes in different physiological and pathological conditions in rabbits or men, are more easily understood on the basis of our findings of the separate cellular localization of allelic specificities than on the basis of the alternative possibility of the coexistence of the two types of molecules in the cytoplasm of every cell. It has in fact been shown, first by Gell and Kelus (20) and then on a more quantitative basis by Rieder and Oudin (21) that the ratio of allelic specificities in isolated antibodies may differ markedly from that found in the total serum gamma globulins of the same individual; if every immunoglobulin-producing cell synthesizes the two kinds of molecules at a given ratio, the above mentioned findings would imply that the antibody-producing cells manufacture side by side specific antibody and "non-specific" gamma globulins and, furthermore, that in the majority of these cells one of the two allelic allotypic specificities is predominant amongst the specific antibody molecules or in the "non-specific" gamma globulins, as the case may be. This is very difficult to accept, and it appears more easy to visualize that in an antigenically stimulated rabbit the

population of plasma cells producing a given antibody may be constituted by members that belong predominantly to one or to another of the two classes of cells marked by the different allotypic specificities; the possible reasons for these postulated differences in the composition of the group of cells engaged in the production of an antibody with respect to that of the global pool of plasma cells, will be discussed later.

Another fact which fits well with our data is the presence of one only of the two allelic allotypes in the gamma G globulins of multiple myeloma in heterozygous humans (22, 23). If the proliferation of plasma cells in these cases is in fact "monoclonal" in that all the neoplastic elements produce the same or very similar globulins (24–26), and if individual normal cells of the plasma cell line in heterozygous humans produce gamma globulins marked with one only of the two allelic specificities, then the above mentioned characteristic of the gamma G globulins of multiple myeloma is readily understood without imputing to the cells of multiple myeloma any basic difference from normal plasma cells with regard to the expression of the genes involved in allotypic specificities.

A third fact, in the field of gamma globulin allotypes, that has been fairly well established, is that in heterozygotes, allelic specificities are found in separate molecules (15–17, 27, 28). In consideration of the presence in each gamma globulin molecule of two L and two H chains, the fact that the two L or H chains always carry the same allotypic specificity even in heterozygotes would require some selective mechanism in the intracellular assembly of these polypeptide chains, if both allelic specificities were present in a single cell. It has in fact been shown recently (29) that there are no apparent restrictions to the pairing of half molecules of gamma G globulins bearing different allotypic markers, and that hybrid molecules are formed at random following recombination *in vitro*. Dray *et al.* (16), Dray and Nisonoff (17), and Gilman *et al.* (15) have put forward the hypothesis that couples of L and H chains may be synthesized in the polyribosomes of the plasma cells and that these chains (bearing identical allotypic specificities) may form pairs immediately. While this may be accepted for the H chains that are in mutual contact in the finished gamma globulin molecule, it is more difficult to visualize a similar mechanism for the L chains (which are those that carry the allotypes determined by the *b* locus, (28) that are widely apart in every model of the gamma globulin molecule that has been proposed thus far (30–32). It is obvious that if instead one only of the two allelic genes is active in a single cell that is producing gamma globulins, as it is indicated by our observations, then even a random assembly of the polypeptide chains would yield symmetrical gamma globulin molecules. Finally, mention should be made of the very interesting observations performed by Dray (33) who has seen a prolonged inhibition of the expression of the paternal allotype in heterozygous baby rabbits born from mothers actively immunized against the same allotype; it would seem possible that, under these circum-

stances, the antibody of maternal origin may inhibit the appearance and/or the proliferation of cells marked by the antigen with which it can react.

Our findings, therefore, contribute to the solution of some problems but, on the other hand, new ones arise. The fact that, with the possible exception of the cells of the germinal centers, immunoglobulins marked by different allotypic specificities controlled by allelic genes are produced by different cells, may at first sight appear to be just one more instance of the general rule whereby the different classes or subclasses of immunoglobulins are produced by different cells (1–4, 34). However it must be pointed out that in these cases we are confronted with a process whereby different *non*-allelic genes are selectively activated (or inactivated) in different cells. This is in good accord with the general law of cellular differentiation, and may be paralleled with other conditions in which very similar cells may, in the same organ, be specialized to produce different proteins at a given moment (see for instance, the production of serum albumin or fibrinogen by different liver cells), (35).

But the allelism of each of the two groups of genes that control the allotypic specificities of, respectively, the L and the H chains of rabbit gamma globulins appears to be well established (36), and therefore the activity of one only of two *allelic* genes in a given plasma cell raises additional questions. Some of these are closely related with the already quoted (20, 21) prevalence of one of the two allelic specificities in isolated antibodies produced by heterozygous rabbits immunized with some "simple" antigens. Since it is unlikely that there may be differences in general functional properties (comparable to those that exist among, for instance, the three main classes of immunoglobulins) between gamma G molecules carrying allelic allotypic specificities, it is also unlikely that the distribution of allelic specificities in isolated antibodies may be the consequence of some general parameters of the immunization process, such as the stage of the immune reaction, the way of administration of the antigen and so on.

It appears, on the other hand, conceivable that it may reflect a selective action by a specific antigen on the immunocompetent cell. This selective action might operate at the cellular or at the subcellular level depending on whether the activation (or inactivation) of one only of the two allelic genes takes place in the cells *before* or *after* their contact with the antigen; a model of the first of these two possibilities might be provided by the clonal selection theory of antibody formation (37), and for the second one by Szilard's (38) explanation of the differentiation of antibody-forming cells. The fact that in the cells of multiple myeloma the inactivation of one of the two allelic genes that control allotypic specificities originates and persists without any demonstrable action by antigenic molecules, might be taken as an indication favoring the first one of the two above mentioned possibilities, but it cannot be excluded that the cells of multiple myeloma derive from elements that have had an antigenic experience.

If selective processes are operating on cells that have already made a decision between allelic allotypes, it is conceivable that other features of the immunoglobulins that they are able to produce may be more important for selection than the allotype itself. In this case one allelic specificity may be considerably in excess of the other amongst molecules of an isolated antibody not because that allotype is more fit than the alternative one to form that particular antibody, but because the antibody-forming cells are the descendants of a limited number (of the order of 10) of progenitor elements amongst which, by chance, the ratio of cells marked by the allelic allotypes was not the same as in the global population of immunologically competent cells.

In our present ignorance of the regulation of the gene activity in mammalian cells, and in particular in antibody-producing cells, it does not seem profitable to speculate further on these problems; it is important, however, to point out that some of them are within the reach of experimentation with the presently available methods.

The Cellular Localization of Allotypes Determined by Non-allelic Genes.—It has been shown (16, 28) that allotypes determined by *non*-allelic genes may be present on the same gamma globulin molecule; in heterozygous it is likely that random association of the non-allelic specificities takes place (28). Our observations with the double staining for two allotypes determined by genes at different loci are consistent with these data inasmuch as they show that in doubly homozygous rabbits most of the plasma cells contain both allotypes and that in heterozygotes all the combinations that have been looked for have been found to be present (Table III) in a percentage of cells not far from that expected on the basis of an independent differentiation of cells with regard to the allotypic specificities determined by genes at different loci.

Demonstration of a^- and b^- Cells.—The presence, in the rabbit, of gamma G globulin molecules that carry only one allotypic specificity has been shown by different authors (15, 17, 19). Our observations show that these "allotype-deficient" gamma G globulins are produced by separate cells; here again there is a discrepancy between our findings and those of Colberg and Dray who found a mixed staining of all plasma cells after double staining with one antiallotype antiserum and horse anti-rabbit gamma G; we have no explanation for this difference, as it can hardly be explained by the fact that the double staining for one allotype and gamma G globulins was performed by Colberg and Dray on the tissues of heterozygous and by ourselves on the tissues of homozygous animals. Bornstein and Oudin (19) define the gamma G molecules that have only one allotypic specificity as a^- or b^- molecules; by analogy we might define the corresponding cells as a^- or b^- cells. The correspondence is good even between the percentages of a^- or b^- molecules with respect to the total gamma globulins that are reported in the literature, and the percentages of a^- or b^- cells amongst gamma globulin-producing cells observed in the spleens of our animals. In fact the relative amount of a^- molecules, that has been studied on

few sera, has been seen to vary (in different animals) from 34 per cent (17) to 14 per cent (15) and we have found that the number of a^- cells was 31 per cent in the spleen of one rabbit (RB 5601) and 12 per cent in that of another (CL 262). The percentage of b^- molecules has been the object of more extensive studies and recently Bornstein and Oudin (19) have established its mean value around 16 per cent in homozygous or in heterozygous rabbits; this value, that is in accord with previous data (15, 17), is also in good accord with the percentages of 14 and 15 per cent of b^- cells (that is cells producing gamma G globulins but lacking allotypic specificity determined by genes at the b locus) observed by us in 2 homozygous rabbits.

Bornstein and Oudin (19) considered three different possibilities to explain the existence of b^- molecules (and obviously the same might be valid for the a^- molecules), these were: (*a*) the b^- molecules carry an as yet undiscovered allotypic specificity controlled by an allelic gene at the b locus; (*b*) they result from the cleavage of gamma G globulins during the storage of the sera or gamma G globulin preparations; and (*c*) the b^- molecules carry B chains (L chains) whose synthesis is controlled by another locus lacking known allelic forms.

The possibility (*a*) is unlikely because b^- molecules are also found in animals that have two different known alleles at the b locus, and our observations obviously rule out the possibility (*b*); if the explanation (*c*) is the correct one, as Bornstein and Oudin are inclined to believe, then the fact that the b^- molecules are produced by separate cells would be more or less analogous to the production by separate cells of immunoglobulins with type I (κ) or type II (λ) L chains as observed by Pernis and Chiappino (3) and by Bernier and Cebra (4).

The Problem of the Germinal Centers.—With the double staining as well as with the staining of consecutive sections with different antisera, the presence of both allelic specificities has always been detected in the same germinal center of spleens and lymph nodes in heterozygous rabbits. The mixture of the two allotypes was uniform and, as far as we can decide on the basis of immunofluorescence, the mixture was present at the level of each of the immunoglobulin-containing "blast" cells in the centers. With regard to these elements, therefore, our observations do not differ from those of Colberg and Dray and may be compared to the mixtures of immunoglobulins with κ or λ chains seen by Pernis and Chiappino (3) in the cells of the germinal centers of human lymphoid tissues.

It might seem, therefore, that the cells of the centers are basically different from those outside in that they are still undecided with respect to the allotypic specificity of the immunoglobulin produced. However the recent demonstration by Miller and Nossal (39) of a fine net of phagocytic cells in secondary as well as in primary lymphoid follicles, raises the possibility that part, at least, of the immunoglobulins revealed by immunofluorescence in the germinal centers may

not be actually produced there, but may be trapped secondarily, perhaps as antigen-antibody complexes. It is obvious that in this case the presence of immunoglobulins synthesized elsewhere might obscure the original pattern of production of allotypic specificity by the "blast" cells of the germinal centers. We have presently under way experiments designed to investigate the origin of immunoglobulins that can be demonstrated by immunofluorescence in the germinal centers of rabbit lymphoid tissues, because the presence at these sites of cells that are undifferentiated with respect to the molecular class or allotype of the gamma globulin molecules that they produce, would have important theoretical implications (3).

SUMMARY

The cellular localization of allotypes in rabbit lymphoid tissues has been studied by immunofluorescence. In heterozygous animals the double staining for two allotypes controlled by allelic genes (A1 and A2; A4 and A5; A4 and A6) has shown the existence of two populations of plasma cells, one containing one allotype and the other the alternative one. The localization in different cells of immunoglobulins marked by allelic allotypic specificities has been confirmed by microspectrography of single cells. An exception to this rule was given by the presence in the germinal centers of lymphoid follicles of apparently uniform mixtures of products of the two allelic genes.

Double staining for two allotypes controlled by genes at different loci showed, instead, the presence of many cells containing both allotypes; the number of these cells was highest in doubly homozygotes, in the other it was consistent with random association of non-allelic specificities.

In addition double staining for one allotype and gamma G globulins in the lymphoid tissues of rabbits homozygous at the *a* or at the *b* locus, has shown the presence of cells containing immunoglobulins that lack one allotype.

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EXPLANATION OF PLATE 56

FIG. 1. Rabbit RB 5247, spleen stained for anti-horse ferritin antibodies: presence, mainly in the periarteriolar sheaths, of plasmablasts and plasma cells positive for antibodies. × 240.

FIG. 2. Rabbit 5530 (genotype A_a^1 A_a^1, A_b^4 A_b^4), spleen stained with rhodamine-conjugated anti-A1 antiserum followed by fluorescein-conjugated anti-A4: the elements of the plasma cell series are stained by both conjugates and appear yellow. × 1400.

FIG. 3. Rabbit 5247 (genotype A_a^1 A_a^1, A_b^4 A_b^6), spleen stained with rhodamine-conjugated anti-A4 antiserum followed by fluorescein-conjugated anti-A6: single cells are stained by one or by the other of the two conjugates. × 1400.

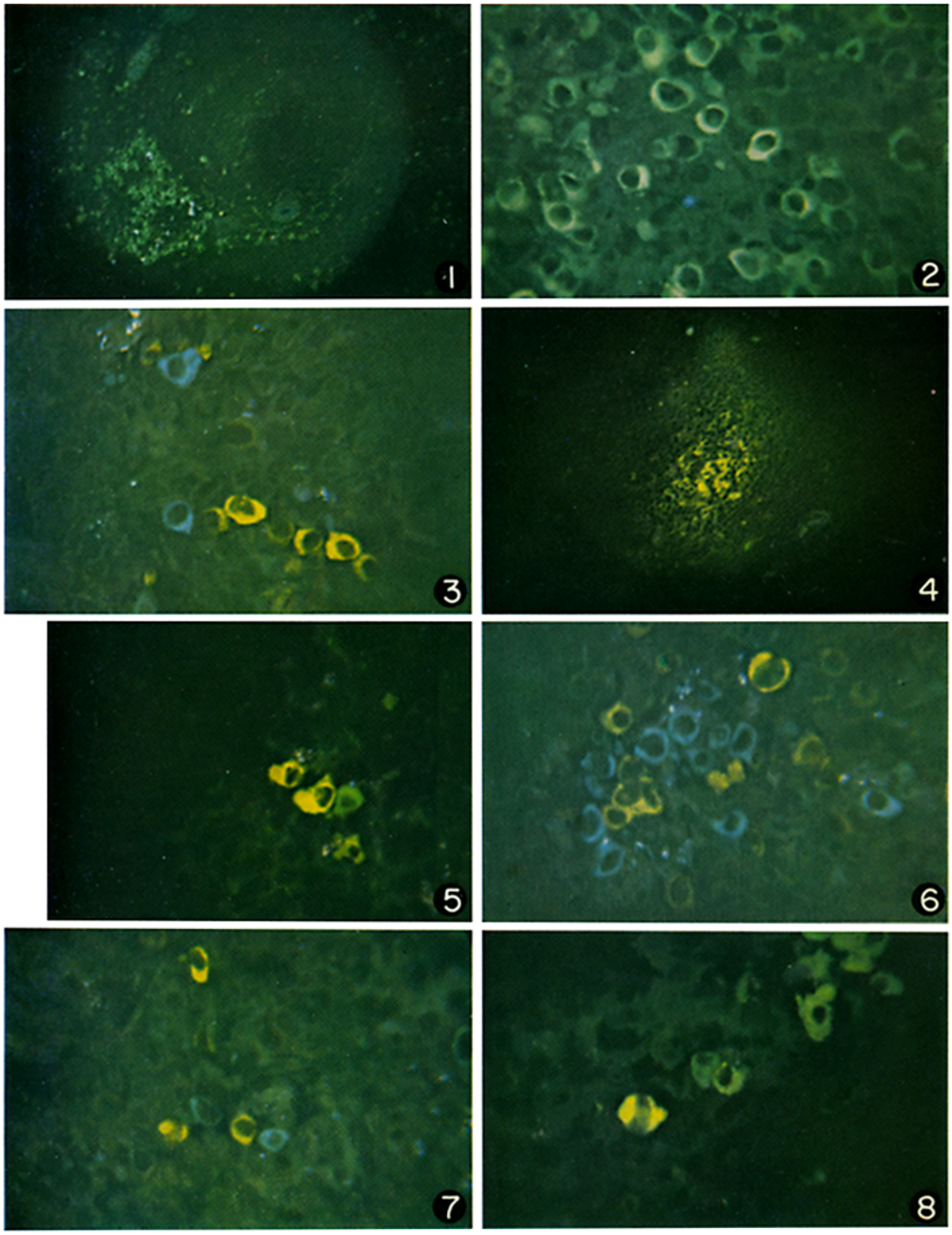
FIG. 4. Same spleen and staining as in Fig. 3: staining of the immunoglobulins in a germinal center by both conjugates. × 240.

FIG. 5. Rabbit CL 262 (genotype A_a^1 A_a^1, A_b^4 A_b^5), spleen stained with rhodamine-conjugated anti-A4 followed by fluorescein-conjugated anti-A5: two plasma cells appear predominantly stained by the anti-A4 and two other by the anti-A5 (see also Text-figs. 3 and 4.) × 1400.

FIG. 6. Rabbit 5549 (genotype A_a^1 A_a^2, A_b^4 A_b^6), spleen stained with rhodamine-conjugated anti-A4 followed by fluorescein-conjugated anti-A6: many cells of the plasma cell line are visible, that have reacted with the anti-A4 *or* with the anti-A6 antibodies. × 1400.

FIG. 7. Same rabbit as in Fig. 6, spleen stained with rhodamine-conjugated anti-A1, followed by fluorescein-conjugated anti-A2; three cells are stained by the anti-A1 and two by the anti-A2. × 1400.

FIG. 8. Rabbit CL 263 (genotype A_a^1 A_a^2, A_b^4 A_b^4), spleen stained with rhodamine-conjugated anti-A4 followed by fluorescein-conjugated anti-gamma G: the majority of the cells appear stained by both conjugates, but one plasma-cell is visible in the center of the figure that was stained only by the anti-gamma G antiserum. × 1400.



(Pernis *et al.*: Cellular localization of immunoglobulins)