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Localization by Immunofluorescence of Gamma-Globulin Allotypes in Lymph Node Cells of Homozygous and Heterozygous Rabbits

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Summary. The cellular production of two rabbit γ-globulin allotypic specificities, A4 and A5, determined by allelic genes was investigated by the fluorescent antibody method. The 7S γ-globulin fractions of precipitating antisera were conjugated to fluorescein isothiocyanate and to lissamine rhodamine B sulphonyl chloride. Frozen sections of lymph nodes from eighteen rabbits, A4–A4 and A5–A5 homozygotes and A4–A5 heterozygotes, were studied after exposure to the fluorescent antibody conjugates. The conjugates, each specific for antigenic determinants of 7S γ-globulin, reacted specifically with the cytoplasm of plasma cells and intrinsic cells of the germinal centres. The rabbit anti-A4 conjugate reacted only with lymph node cells of A4–A4 and A4–A5 rabbits; the rabbit anti-A5 conjugates reacted only with cells of A5–A5 and A4–A5 rabbits; the horse anti-rabbit γ-globulin conjugates reacted with cells of all three genotypes. By a variety of techniques, identical cellular localization of the two allotypes, A4 and A5, was found in the A4–A5 heterozygotes. Less than 1 per cent of the cells in any heterozygous lymph node section contained one allotype without the other.

INTRODUCTION

Antigenic specificities present on proteins in some normal individuals, but not on the corresponding proteins in other individuals of the same species, are called 'allotypic specificities' (Oudin, 1956). This phenomenon of 'allotypy' has been found to exist for α-, β- and γ-globulins (Dray and Young, 1958). The allotypic specificities of γ-globulins are of particular interest because of the central role of γ-globulins in immunological reactions.

Although allotypy has been found in other species, including man, the 7S γ-globulin allotypes of the rabbit are at present the most clearly defined. The 7S γ-globulins of individual rabbits are antigenic for other rabbits and yield precipitating antibodies (Dray and Young, 1958, 1959; Dubiski, Dudziak and Skalba, 1959; Dubiski, Dubiska, Skalba and Kelus, 1961; Oudin, 1960a, b). These isoprecipitins identify allotypic (antigenic) specificities present on γ-globulin molecules in the sera of some, but not all rabbits. Allotypic specificities A1, A2 and A3 are controlled by three alleles at one genetic locus, *a*; allotypic specificities A4, A5 and A6, by three alleles at a second locus, *b* (Dray, Dubiski,

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Kelus, Lennox and Oudin, 1962). The *a* and *b* loci are not closely linked (Dray, Young and Gerald, 1963a). A new allotypic specificity '*P*' is determined at a third locus (Dray *et al.*, 1963a).

Since a variety of genetically defined antigenic forms (allotypes) of γ-globulin coexist in the same individual, it became apparent that immunochemical analysis of the γ-globulins and immunofluorescent studies of cells containing them would yield meaningful information concerning the nature of genetic control of protein synthesis. Immunochemical studies of this relationship are presented elsewhere (Dray and Nisonoff, 1963; Dray, Young and Nisonoff, 1963b). This paper presents immunofluorescent studies of the contribution of allelic genes, Ab^4 and Ab^5, to the synthesis of γ-globulin within individual cells of homozygous and heterozygous rabbits.

MATERIALS AND METHODS

Animals

Flemish giant and New Zealand white rabbits were obtained from closed colonies. The rabbits used in this study were progeny of parents with known allotypic specificities (except for a Race III rabbit, 38V3, which was obtained from the Roscoe B. Jackson Memorial Laboratory and used for immunization). The progeny were bled from the ear at 8–10 weeks of age. The sera were typed for the known allotypic specificities with reference antisera in micro-Ouchterlony gel diffusion plates or in Oudin tubes (Dray *et al.*, 1963a).

Preparation of γ-Globulin

After dialysis against buffer, serum was chromatographed on a diethylaminoethyl–cellulose column according to the method of Levy and Sober (1960) for the separation of 7S γ-globulin from whole serum. Serum from one or several rabbits of the same allotype was placed on a 45 cm. column of DEAE cellulose (0·4 ml. serum per g. DEAE) packed under 10 lb./in.^{2} pressure and was eluted with phosphate buffer (0·008 M at pH 8·0 or 0·017 M at pH 6·4) at 4°. Chromatographic separation was followed by determination of the optical density at 280 mμ.

The first protein peak was concentrated by vacuum ultrafiltration at 20 lb./in.^{2} using Visking cellulose dialysis tubing. The purity of the 7S γ-globulin was established by electrophoresis in agar gel and by immunoelectrophoresis against reference sheep antisera to rabbit serum.

Antisera

Antisera were prepared by immunization of rabbits with γ-globulin emulsified in Freund's adjuvant. The recipient rabbits lacked only one known allotypic specificity present in the γ-globulin of the donor rabbit. The antisera were tested for specificity by agar gel methods (Dray *et al.*, 1963a).

The horse anti-rabbit γ-globulin (kindly supplied by Dr. A. M. Pappenheimer, Jr.) had been prepared by immunization with a purified alcohol-fractionated γ-globulin. Immunoelectrophoresis demonstrated antibodies to γ-globulin of both A4–A4 and A5–A5 homozygotes; a faint precipitin arc against siderophilin was also present. The horse antiserum precipitated 99 per cent of ^{131}I-labelled 7S γ-globulin (quantitative analysis by Dr. A. Nisonoff).

Preparation of Conjugates

In order to reduce the amount of non-specific staining which occurs with fluorescent antibody methods (Coons and Kaplan, 1950), a DEAE cellulose prepared 7S γ-globulin fraction of each of the antisera was used for conjugation to fluorescent dyes. Micro-Ouchterlony tests showed that specific antibody activity was retained. These were conjugated with fluorescein isothiocyanate, by the method of Marshall, Eveland and Smith (1958). Fluorescein isothiocyanate was obtained as an amorphous preparation (Sylvana Chemical Co.) and as a crystalline preparation (Baltimore Biological Laboratories). A dye to protein ratio of 1 : 20 by weight was used. The dry fluorescein powder was reacted in a spinner flask for 18 hours at 4° with the protein solution buffered at pH 9·0.

Gamma-globulin fractions of antisera were conjugated to lissamine rhodamine B 200, according to the method of Chadwick, McEntegart and Nairn (1958). The dye was obtained dextrin-free (Imperial Chemicals Industries Ltd.). Lissamine rhodamine B 200 sulphonyl chloride was added slowly as a solution in acetone with constant stirring of the reactants for a period of 60–90 minutes at 4°.

TABLE 1

ALLOTYPIC SPECIFICITIES AND GENOTYPES OF RABBITS USED FOR IMMUNOFLUORESCENT STUDY OF LYMPH NODES

Rabbits	*Allotypic specificities present*		*Genotype*	
	a *locus*	b *locus*	a *locus*	b *locus*
A4–A4 homozygotes				
34FZ-3	A1	A4	Aa^1/Aa^1	Ab^4/Ab^4
AZ89	A1	A4	Aa^1/Aa^1	Ab^4/Ab^4
BBK	A3	A4	Aa^3/Aa^3	Ab^4/Ab^4
ZX21	A3	A4	Aa^3/Aa^3	Ab^4/Ab^4
ZX33	A3	A4	Aa^3/Aa^3	Ab^4/Ab^4
20FZ-5	A1,A3	A4	Aa^1/Aa^3	Ab^4/Ab^4
A5–A5 homozygotes				
FLZ	A2	A5	Aa^2/Aa^2	Ab^5/Ab^5
CG72-1	A1,A2	A5	Aa^1/Aa^2	Ab^5/Ab^5
FLI35	A1,A2	A5	Aa^1/Aa^2	Ab^5/Ab^5
41FZ-1	A1,A2	A5	Aa^1/Aa^2	Ab^5/Ab^5
A4–A5 heterozygotes				
CG693-4	A2	A4,A5	Aa^2/Aa^2	Ab^4/Ab^5
CF990-2	A2	A4,A5	Aa^2/Aa^2	Ab^4/Ab^5
CF69-3	A2	A4,A5	Aa^2/Aa^2	Ab^4/Ab^5
CG503-1	A1,A2	A4,A5	Aa^1/Aa^2	Ab^4/Ab^5
CH434-1	A1,A2	A4,A5	Aa^1/Aa^2	Ab^4/Ab^5
CH242-4	A2,A3	A4,A5	Aa^2/Aa^3	Ab^4/Ab^5
CF677-1	A2,A3	A4,A5	Aa^2/Aa^3	Ab^4/Ab^5
20FZ-2	A1,A3	A4,A5	Aa^1/Aa^3	Ab^4/Ab^5

After conjugation, each γ-globulin–fluorochrome conjugate was separated from free dye by gel filtration with Sephadex G-25, medium grade (Pharmacia). Rapid separation of conjugate from free dye was effected with almost no dilution (Killander, Pontén and Rodén, 1961).

Non-specific staining was rare; if it occurred, the conjugate was absorbed one to three times with mouse liver powder according to the method of Coons, Leduc and Connolly (1955).

Preparation of Tissue Sections

Tissues were obtained from normal rabbits: six A4 homozygotes, four A5 homozygotes and eight A4–A5 heterozygotes. These eighteen rabbits are listed with their allotypic specificities in Table 1. Allotypic specificities A1, A2 and A3, determined at the *a* locus, were present in the A4–A4 homozygote group; A1 and A2, but not A3 were present in the A5–A5 homozygote group.

Small tissue blocks were frozen quickly in petroleum ether at about −60° according to the method of Tobie (1958). After blotting quickly, they were wrapped in 'Saran' plastic film and aluminum foil on a block of solid carbon dioxide, and stored at −60°. Generally, tissues were used within 2 weeks to minimize autofluorescence.

Lymph nodes were chosen for study because their structure is better defined than spleen, and background staining is less prominent. Mesenteric nodes were more frequently used because γ-globulin-containing cells were more numerous than in the popliteal nodes of our cage-reared rabbits.

The tissues were sectioned in a Harris cryostat (−15° to −20°) as described by Coons, Leduc and Kaplan (1951). The limit of reproducible sectioning was 3 μ thickness.

Sections were dried by fan and *in vacuo* at room temperature for at least 45 minutes. After drying briefly, sections from adjacent portions of the node were placed in 10 per cent formalin buffered at pH 7·0 and stained with haematoxylin and eosin, or placed in absolute ethanol and stained with methyl green–pyronin stain (Aloe).

Sections for fluorescent-antibody studies were fixed for 10 minutes in acetone at room temperature and then dried in a vacuum chamber for at least 15 minutes. Lymph node sections so treated could be stored *in vacuo* at 4° for as long as 2 days before autofluorescence became too great. Routinely, tissues were sectioned, fixed with acetone, and reacted with the fluorescent conjugates on the same day.

Fluorescent Staining

Sections were reacted with fluorescent conjugates according to the method of Coons and Kaplan (1950). Tissue sections were flooded with the fluorescent antibody conjugate for 10–30 minutes at room temperature under a Petri dish containing moistened filter paper. The excess conjugate was washed off by two changes of pH 7·0 phosphate buffered isotonic saline with gentle agitation, wiped clear, removing as much saline from the tissue section as possible before mounting in 10 per cent of buffered saline in glycerol.

Sequential staining of tissue sections with both fluorescein- and rhodamine-labelled antibodies was done in a similar manner. A fluorescein–antibody conjugate was generally applied before a rhodamine–antibody conjugate because fluorescein could be seen readily beneath a second layer of rhodamine-labelled antibody. The reverse order was also satisfactory but not as clearly evident. The effect of the second fluorochrome was enhanced when it reacted with the antigenic sites of the first in a manner similar to that of any indirect fluorescent antibody method (Weller and Coons, 1954). As is evident from Table 2, the conjugates employed in this study had both an antigenic and an antibody specificity reacting in this system, i.e. anti-A4 was A5 γ-globulin and anti-A5 was A4 γ-globulin.

Reaction times employed were 20–30 minutes for the first conjugate followed by 10 minutes of saline wash. After removing as much saline as possible, the second conjugate was allowed to react for 10–20 minutes, then rinsed off. In each experiment, one tissue section was reacted with anti-A4 fluorescein followed by anti-A5 rhodamine and a companion section reacted with anti-A5 fluorescein followed by anti-A4 rhodamine. Four

control sections were each flooded with one of the fluorescent antibody reagents; anti-A4 fluorescein, anti-A5 fluorescein, anti-A4 rhodamine, or anti-A5 rhodamine, applied in the same dilution for an equal length of time as to the doubly stained section. This provided assurance that both fluorescent antibodies could be seen on a section stained with the two fluorochromes.

TABLE 2

FLUORESCENT ANTIBODY CONJUGATES EMPLOYED FOR IMMUNOFLUORESCENT STUDY OF LYMPH NODES

	Antigenic specificity	*Antibody specificity*	*Fluorochrome*
1	A4	Anti-A5	Fluorescein
2	A4	Anti-A5	Rhodamine
3	A5	Anti-A4	Fluorescein
4	A5	Anti-A4	Rhodamine
5	Horse γ-globulin	Anti-rabbit γ-globulin	Fluorescein
6	Horse γ-globulin	Anti-rabbit γ-globulin	Rhodamine

Fluorescence Microscopy

A Leitz Orthomat microscope was used with a cardioid immersion type darkfield condenser. One light source used was that described by Tobie (1958), but for the main part, the Osram HBO-200 high pressure vapour lamp was used.

Ultraviolet at 360–370 mμ was used because it gave diminished background fluorescence of the tissue and better colour discrimination when used with a nearly colourless eyepiece filter. A BG38 heat-absorbing filter, a Jena glass UG-1 exciting filter, and a Wratten 2A eyepiece ultraviolet absorption filter were used.

The microscope stage was fitted with a graduated micrometer for localization of specific areas and for mapping. Photographs were taken on Superanscochrome daylight type 35 mm. film using an attached Leitz Orthomat camera with automatic exposure timing.

RESULTS

SPECIFICITY OF ANTIBODY CONJUGATES

Rabbits were obtained from controlled breedings in a closed colony and were typed against reference antisera for the known γ-globulin allotypes. A pure 7S γ-globulin preparation with only one of its known allotypic specificities lacking in the recipient was used for immunization. The antisera were tested by immunoelectrophoresis and showed precipitin bands only to the 7S γ-globulin of the donor and reference sera.

Each 7S γ-globulin antibody conjugate was tested against A4 and A5 7S γ-globulin by the micro-Ouchterlony agar-gel method. The anti-A4 conjugates reacted with A4 γ-globulin but not with A5 γ-globulin, and the anti-A5 conjugates reacted with A5 but not with A4 γ-globulin.

The fluorescein and rhodamine conjugates listed in Table 2 were used to stain sections of lymph nodes removed from A4–A4 homozygous, A5–A5 homozygous and A4–A5 heterozygous rabbits. Table 3 outlines the reactions of these antibody conjugates.

When the anti-A4 fluorescein or rhodamine conjugate was reacted with a lymph node

from an A4–A4 homozygote, specific green or orange-red fluorescence was observed in the cytoplasm of cells in the plasma cell series and of intrinsic cells of the germinal centres. When the anti-A4 conjugates were reacted with a lymph node section from an A5–A5 homozygote, no fluorescence was present. The reaction with a section from an A4–A5 heterozygote could not be distinguished from that with a section from an A4–A4 homozygote.

TABLE 3

SPECIFIC STAINING REACTIONS OF CONJUGATES WITH CELLS OF HOMOZYGOUS AND HETEROZYGOUS LYMPH NODE SECTIONS

Tissues	*Conjugates*					
	Anti-A4		*Anti-A5*		*Horse anti-RGG*	
	Fluorescein	*Rhodamine*	*Fluorescein*	*Rhodamine*	*Fluorescein*	*Rhodamine*
A4–A4 homozygote	+	+	0	0	+	+
A5–A5 homozygote	0	0	+	+	+	+
A4–A5 heterozygote	+	+	+	+	+	+
A4–A4 homozygote + anti-A4 antiserum	0(±)	0(±)	+(i)	+(i)	ND	ND
A5–A5 homozygote + anti-A5 antiserum	+(i)	+(i)	0(±)	0(±)	ND	ND
A4–A4 homozygote + anti-A5 antiserum	+	+	0	0	ND	ND
A5–A5 homozygote + anti-A4 antiserum	0	0	+	+	ND	ND

RGG: rabbit γ-globulin.
+: specific cellular fluorescence; 0: no fluorescence; 0(±): no fluorescence or marked inhibition of fluorescence; i: indirect staining; ND: not done.

The anti-A5 conjugates reacted with cells from the A5–A5 homozygote, but did not react with cells from the A4–A4 homozygote. The A4–A5 heterozygotes could not be distinguished from the A5–A5 homozygotes by the reactions of the anti-A5 conjugates with their tissues. Thus the anti-A4 and the anti-A5 fluorescein and rhodamine conjugates demonstrated specificity of reaction in agar gel and in sections of lymph nodes from ten homozygous rabbits, as summarized in Table 3. Characteristic specific green fluorescence with anti-A4-Fl and orange-red fluorescence with anti-A5-Rh are seen in Figs. 3 and 4.

A horse antiserum to rabbit γ-globulin was tested by agar gel immunoelectrophoresis against sera from both an A4–A4 homozygote and an A5–A5 homozygote. A single dense precipitin arc developed against γ-globulin of each serum, as well as a faintly visible arc against siderophilin. The γ-globulin fraction of this antiserum was divided into two portions; one was conjugated with fluorescein and the other with rhodamine. These two conjugates reacted with the A4–A4, A5–A5, and the A4–A5 lymph nodes to produce in all three specific cytoplasmic fluorescence; cellular localization was similar to that of the anti-allotype conjugates.

The specificity of the anti-allotype and the anti-rabbit γ-globulin (anti-RGG) conjugates was tested by inhibition procedures. A4–A4 lymph node sections were exposed to anti-A4 antiserum prior to layering with anti-A4 conjugate; fluorescence was either completely abolished or markedly inhibited. Similarly, exposure of an A5–A5 section to anti-A5 antiserum prior to the anti-A5 conjugate caused inhibition of staining.

However, the substitution of anti-A5 antiserum for anti-A4 antiserum or of anti-A4

antiserum for anti-A5 antiserum failed to prevent reaction between the conjugated antibodies and their corresponding antigens in the tissues. Furthermore, when A4–A4 lymph nodes were exposed to anti-A5 fluorescein prior to layering with anti-A4 rhodamine, characteristic specific orange-red fluorescence was observed. Similarly, exposure of A5–A5 lymph nodes to anti-A4 fluorescein did not interfere with the specific staining with anti-A5 rhodamine.

The horse anti-RGG conjugates were inhibited by prior exposure of lymph node sections to unconjugated horse anti-RGG antiserum. However, prior exposure to horse anti-RGG antiserum did not inhibit to the same extent the reaction with an anti-allotype conjugate.

Selection of the appropriate reactants produced indirect staining of cells containing γ-globulin allotypes. As shown in Table 2, anti-A4 antibodies have A5 antigenic sites and anti-A5 antibodies have A4 antigenic sites. Thus anti-allotype antibodies can function as the middle layer in a 'fluorescent sandwich'. When A4–A4 sections were exposed first to anti-A4 antiserum, exposure to anti-A5 conjugate produced cellular fluorescence. When A5–A5 sections were exposed first to anti-A5 antiserum exposure to anti-A4 conjugate produced cellular fluorescence. The same reactions occurred when conjugated γ-globulin fractions of antiserum were substituted for whole antiserum in the 'sandwich'. When two different labels were used, the fluorescent label of the top layer was generally more prominent than the label of the middle layer.

Although fluorescein conjugates are brighter than rhodamine conjugates because of the greater quantum efficiency of the fluorescein dye, both types of conjugates appeared equally efficient in their reactions with antigenic sites in cells of the tissue section. When an A4–A4 homozygous lymph node section was first stained with anti-A4-rhodamine conjugate, staining with the anti-A4-fluorescein conjugate failed to light up new cells or groups of cells; when the fluorescein conjugate was used first followed by the rhodamine, again no new cells were seen. Similar results occurred in the anti-A5 system.

LOCALIZATION OF THE γ-GLOBULIN ALLOTYPES

The number of lymph node cells which were fluorescent was estimated to be less than 10 per cent in any section examined. Localization of fluorescence in the eighteen rabbit lymph nodes studied did not differ in homozygous lymph nodes stained with the appropriate anti-allotype conjugate, heterozygous lymph nodes stained with either anti-A4 or anti-A5 conjugates, or homozygous and heterozygous lymph nodes stained with horse anti-RGG conjugates.

Two major sources of γ-globulin allotypes were found. The first was small groups and cords of mature and immature plasma cells in the medulla, frequently situated along vessels and trabeculae of the node. The cells in these groupings were: typical mature plasma cells with eccentric nuclei and a low nucleus–cytoplasm ratio; less mature plasma cells with larger nuclei located in the midst of the cytoplasm; and large cells, predominantly nucleus with only a very thin rim of fluorescent cytoplasm. These last cells were probably plasmablasts (haemocytoblasts, transitional cells), but differentiation from medium and large lymphocytes could not be made with certainty. Very infrequent but striking when seen were plasma cells with brilliantly fluorescent Russell bodies (Fig. 10). The fluorescent characteristics of these were the same as those found with anti-γ-globulin conjugates (White, 1954).

The germinal centres of the follicles were the second major source of the γ-globulin allotypes. Fluorescence was found in the cytoplasm of cells in the central portion of the follicles. Most of these cells possessed only a thin rim of fluorescent cytoplasm and appeared to be predominantly immature plasma cells and blast cells, but again absolute differentiation from medium and large lymphocytes could not be made. The mature small lymphocytes which constitute most of the outer portion of such germinal centres and the major cell type of the node did not demonstrate fluorescence in any of the sections. Fluorescence was also seen in a few cells scattered around the periphery of the follicle; these could be identified as mature and immature plasma cells.

The popliteal nodes examined from unimmunized rabbits demonstrated γ-globulin allotypes predominantly in cells of the lymphoid germinal centres and relatively little in the medullary portion of the node. The medullary formation of γ-globulin allotypes was more pronounced in the mesenteric nodes and a greater number of the plasma cell series were present. The large size of the mesenteric nodes and the greater number of fluorescent cells suggested chronic stimulation by antigens from the gut.

Fluorescence was usually diffusely localized throughout the cytoplasm; occasionally it appeared as small globules of varying size. The tiny particles may be artefacts, although both types of fluorescence were seen in the same section. Some cells had a small 'flare' of fluorescence in the nucleus. Discrete, well-outlined homogeneous areas of fluorescence were seen that were not obviously surrounding a dark nucleus. These areas may represent secreted allotypic γ-globulin or intracytoplasmic γ-globulin of cells, the nuclei of which were not in the section examined. Occasional cells appeared to be in the process of cytoplasmic fragmentation or degeneration; this may represent holocrine secretion of allotypic γ-globulin (Ortega and Mellors, 1957).

Popliteal nodes from rabbits immunized by two footpad injections of ovalbumin or of diphtheria and tetanus toxoid differed from unimmunized popliteal nodes; more fluorescence was present, both intracellular and extracellular, the grouping of plasma cells was less distinct and a characteristic diffuse fluorescence was present in granulocytes. This was similar to the non-specific fluorescence of granulocytes found by others (Nairn, 1962).

OBSERVATIONS ON CONSECUTIVE SECTIONS

To compare the localization in heterozygotes of the two allotypes, A4 and A5, determined by alleles at the *b* locus, consecutive 3 and 4 μ frozen sections of lymph nodes were prepared. Each of a pair of consecutively cut sections was oriented in the same manner on an individual slide. One section was stained with anti-A4 conjugate and the other was stained with anti-A5 conjugate. Generally, sections stained with anti-A4 showed more intense fluorescence than sections stained with anti-A5. Since over twice as much A4 as A5 γ-globulin is present in the serum of the A4–A5 heterozygote, 20 FZ-2 (Dray and Nisonoff, 1963), the same ratio probably exists in the node. By means of the microscope stage micrometer, the architecture of each section was plotted on graph paper and the localization of fluorescent cells compared. Although sections were cut at the 3 μ setting of the microtome, differences in appearance between the two paired sections were sufficient to make comparison of individual cells quite difficult by ultraviolet dark-field microscopy. Nonetheless, it was possible to compare with accuracy the specific fluorescence in most of the germinal centres of the paired sections and in several of the medullary groups of fluorescent cells. By photographic transparencies, it was possible in a few paired sections to

identify both the A4 and the A5 allotypic γ-globulin within the cytoplasm of a single cell. Paired sections are shown in Figs. 12 and 13. The presence of both allotypes could be identified in at least twenty cells.

To determine whether the minor differences in pattern which existed between the paired sections reflected a difference in cellular distribution, eight consecutive sections were cut at an indicated thickness of 3 μ from a single node of rabbit 20FZ-2. Sections 1, 2, 5 and 6 were stained with anti-A4 conjugate; sections 3, 4, 7 and 8 were stained with anti-A5 conjugate. The differences between adjacent sections stained with the same conjugate were comparable to those between adjacent sections stained with different conjugates. Staining adjacent sections with anti-allotype conjugate and with the horse anti-γ-globulin conjugate gave similar results.

RESTAINING EXPERIMENTS

An experimental method of reacting an identical area with two conjugates of different specificity was suggested by the work of White (1958). A lymph node section from rabbits CH434-1 and CF693-4 was reacted with anti-A4 fluorescein and a companion section from each reacted with anti-A5 fluorescein. Eight groups of fluorescent plasma cells were localized and a photograph of each taken. Most of the groups were then exposed to ultraviolet light (400 mμ) for 30 minutes; this diminished the fluorescence markedly.

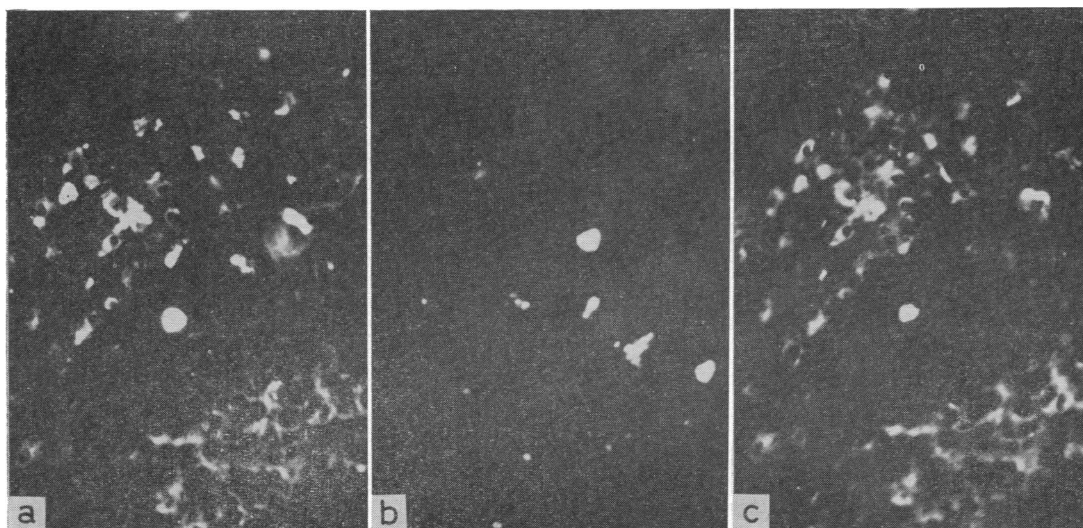

FIG. 1. The same area of a lymph node section from an A4–A5 heterozygous rabbit CH434-1 photographed after reaction in turn with anti-A4 fluorescein (a); with ultraviolet light (390–410 mμ) for 30 minutes (b); and with anti-A5 fluorescein (c). Fig. 1(a) demonstrates specific cellular fluorescence with anti-A4. Fig. 1(b) shows the loss of specific fluorescence due to the fatiguing effect of ultraviolet light on fluorescein. Only the white autofluorescent matter is seen. Fig. 1(c) shows that, although the same cells again are fluorescent, no new cells are stained by the second conjugate.

Controls demonstrated that this dosage did not interfere with subsequent reaction of an allotype with its antibody conjugate. The sections were then reacted with the opposite conjugate, either fluorescein- or rhodamine-labelled anti-A5 or fluorescein- or rhodamine-labelled anti-A4. The same areas were again localized and photographed. Of the eight

areas photographed, only two areas showed cells (a total of three cells) which fluoresced after the second conjugate but had not fluoresced after the first. These three cells constituted less than 2 per cent of the cells studied. While it is conceivable that these three cells were making only one of the allotypic γ-globulins, the possibility could not be ruled out that the second conjugate was reacting with poorly-conjugated antibody overlying the cytoplasm of these cells after the first staining procedure. This experiment is illustrated in Fig. 1.

DOUBLE STAINING EXPERIMENTS

The availability of fluorochromes of contrasting colour and suitable methods for conjugating them to specific antibodies provided an approach for obtaining more precise information about individual cells and their content of the types of γ-globulin.

As can be seen in Table 2, each conjugate is capable of reacting in this system specifically both as an antibody and as an antigen. This complicated the double staining procedure since the second conjugate not only reacted with antigenic determinants on γ-globulin in the cells but also with antigenic determinants on the first conjugate.

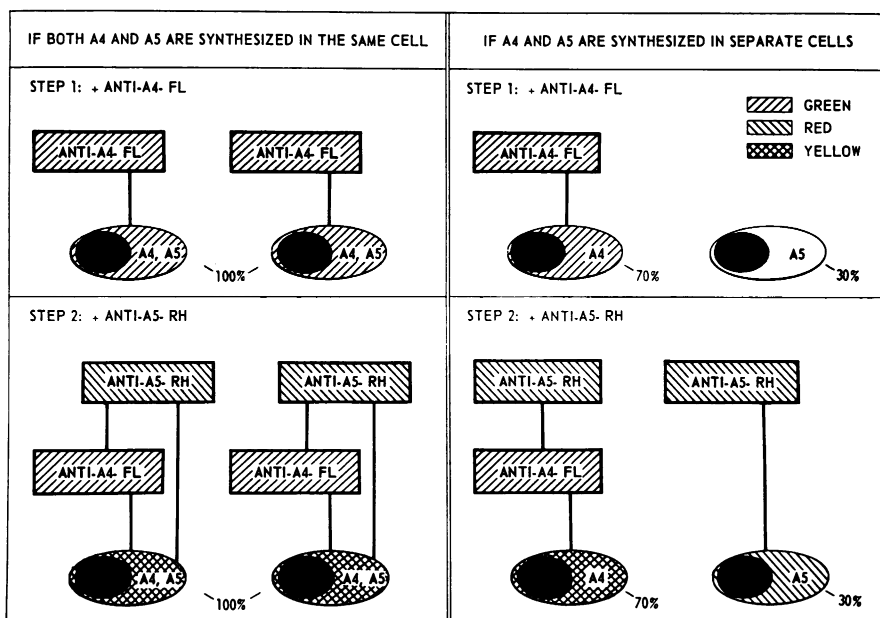

Fig. 2. Two hypotheses of cellular production in the Ab^4/Ab^5 heterozygous rabbit of γ-globulin allotypes A4 and A5 tested by the double staining method with anti-A4 fluorescein conjugate and anti-A5 rhodamine conjugate. In step 1, cells containing A4 are labelled with the green fluorescence of fluorescein. In step 2, cells containing A5 alone are labelled with the orange-red fluorescence of rhodamine. Cells previously labelled with the A5 anti-A4 fluorescein conjugate are labelled yellow by the addition of the A4 anti-A5 rhodamine. If the two allotypes are present in separate cells, 30 per cent of fluorescent cells should fluoresce orange-red. Percentages are based on the findings of Dray and Nisonoff (1963).

Fig. 2 indicates graphically the approach to the problem taken in the experiments using two conjugates of contrasting colour. When the green fluorescence of a conjugate is present together with the red fluorescence of a rhodamine conjugate in a cell or in a tissue structure, the resultant fluorescence is yellow and can be readily distinguished (Hiramoto, Bernecky and Jurand, 1962).

The diagram illustrates the two exclusive hypotheses: (1) the two allotypic γ-globulins are synthesized in the same cell; (2) the two allotypic γ-globulins are synthesized in separate cells, never in the same cell.

In Fig. 2, anti-A4–fluorescein conjugate, flooded over the tissue section, reacts with A4 γ-globulin present in some of the lymph node cells; these cells show green fluorescence of the cytoplasm (Fig. 3). After the first conjugate is rinsed off, anti-A5 rhodamine conjugate is allowed to react with the section. This reacts not only with A5 γ-globulin present in some of the cells of the section, but also with the A5 antigenic sites of the molecules of the fluorescein–antibody conjugate which have reacted with A4 γ-globulin in the cells, as shown in Fig. 2 by the vertical lines representing specific antigen–antibody reactions.

The second step differentiates between the two hypotheses. If both allotypes are found in the same cell, then all cells fluorescing should be yellow, as a result of the presence of both fluorescent conjugates. If each allotype is to be found in a separate cell, then not only would some cells be yellow, the result of anti-A4-Fl reacting with the A4 in the cytoplasm and of anti-A5-Rh reacting with the A5 antigenic sites on the antibody molecules of the first conjugate, but also a significant number (30 per cent) of cells will fluoresce orange-red. Only the anti-A5-Rh would react with the 30 per cent of cells containing A5 γ-globulin alone.

The two hypotheses were also tested in the reverse manner, employing first anti-A5-Fl, then anti-A4-Rh.

The percentages in Fig. 2 refer to the finding (Dray and Nisonoff, 1963) that in an A4–A5 heterozygote (20FZ-2), studied by precipitations of ^{131}I-labelled γ-globulin with anti-A4 and anti-A5 antisera, 64 per cent of 'pure' 7S γ-globulin molecules had A4 antigenic determinants and 27 per cent had A5 antigenic determinants. There were no molecules of mixed A4 and A5 specificity. Since this added up to 91 per cent, 9 per cent of the γ-globulin molecules remain unaccounted for; these molecules might have specificities controlled by genes at other loci.

If it is assumed (the second hypothesis) that some cells make A4 γ-globulin and others make A5 γ-globulin, the ratio of cells producing A4 or A5 γ-globulin should be the same ratio as that of the serum allotypes, provided each cell produces the same amount of allotypic γ-globulin. Thus, in the first experiment (Fig. 2), if the allotypes are made in separate cells, then 30 per cent of all the fluorescent cells should be orange-red, the colour of the anti-A5-Rh alone.

In the reverse experiment, employing first the anti-A5-Fl, then anti-A4-Rh, 70 per cent of the fluorescent cells should fluoresce orange-red, the colour of the anti-A4-Rh alone.

In most of the doubly stained sections a few cells were found showing only the orange-red fluorescence of rhodamine and no evidence of the fluorescein conjugate. This fluorescence was sharply localized to the cytoplasm, leaving the nucleus dark. Fig. 11 shows such a cell with orange-red fluorescence. These cells were estimated to constitute less than 1 per cent of the fluorescent cells in any of the sections examined; they generally ranged from none to 0·5 per cent. The number of such cells was thus insufficient to support the hypothesis that A4 and A5 γ-globulin are generally produced in separate cells. These cells with orange-red fluorescence stood out sharply in contrast to the yellow fluorescence of nearly all of the fluorescent cells in a section reacted with conjugates of contrasting fluorochromes. They appear to represent rare exceptions to the general conclusion that A4 and A5 γ-globulin are produced in the same cell in the heterozygous rabbit.

The results obtained from the two double staining methods were the same for lymph

nodes from all eight A4–A5 heterozygous rabbits. Results did not differ when anti-A4-Fl was followed by anti-A5-Rh or when anti-A5-Fl was followed by anti-A4-Rh. Virtually all fluorescent cells were yellow, a colour resulting from the presence of both the green fluorescein-labelled antibody and the red rhodamine-labelled antibody. The vast majority of these cells had a diffuse yellow fluorescence confined to the cytoplasm. Usually this colour did not vary from area to area in a section or from cell to cell or within a single cell. There were no cells which had discrete green and red areas of the cytoplasm. This can be attributed to reaction of the rhodamine antibody conjugate with the antigenic sites on molecules of the fluorescein conjugate. The yellow fluorescence found in almost all of fluorescent cells in the double staining experiments is shown in Figs. 5–8.

In some lymph node sections there were a few groups of plasma cells in the medullary portion of the node which showed a variation in colour from cell to cell, ranging from predominantly yellow-green to predominantly orange-red, but not the colour of either conjugate alone. This range of staining is seen in the group of plasma cells in Fig. 9. This range of colour between different cells suggests that the individual cell ratio of the two allotypes may differ from the ratio found in the serum. However, it is conceivable that this range may be due at least in part to technical factors, since the staining with the second conjugate is complex, being both direct and indirect.

Individual cells were occasionally found which had a range of fluorescence from yellow to almost orange-red within their cytoplasm, suggesting that A4 and A5 γ-globulin may be produced in different areas of the cytoplasm in these cells.

Sections of lymph nodes from three A4–A5 heterozygotes were reacted with two conjugates of contrasting fluorescence: either anti-A4 fluorescein or anti-A5 fluorescein was used in the first step; horse anti-RGG rhodamine was used in the second step. The same types of controls were used. As shown in Fig. 14, characteristic yellow fluorescence demonstrating the presence of the two conjugates of contrasting colour was found with rare exception in all fluorescent cells. Thus, the anti-A4-Fl and the anti-A5-Fl react with the

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Figs. 3–8. Photomicrographs of A4–A5 heterozygous lymph nodes were taken using dark field illumination with ultraviolet light at 360–370 mμ. Superanscochrome daylight type 35 mm film was employed with exposure times ranging from 1 to 7 minutes.

Fig. 3. Rabbit CG503-1. Germinal centre cells and plasma cells, reacted with anti-A4 fluorescein conjugate, demonstrate green fluorescence. This section came from the same lymph node as the sections shown in Figs. 4–5, and was sectioned, fixed, and reacted with a fluorescent conjugate on the same day. The white and blue spots seen in this and other photographs are due to auto-fluorescence of foreign matter and tissue, respectively. ×300.

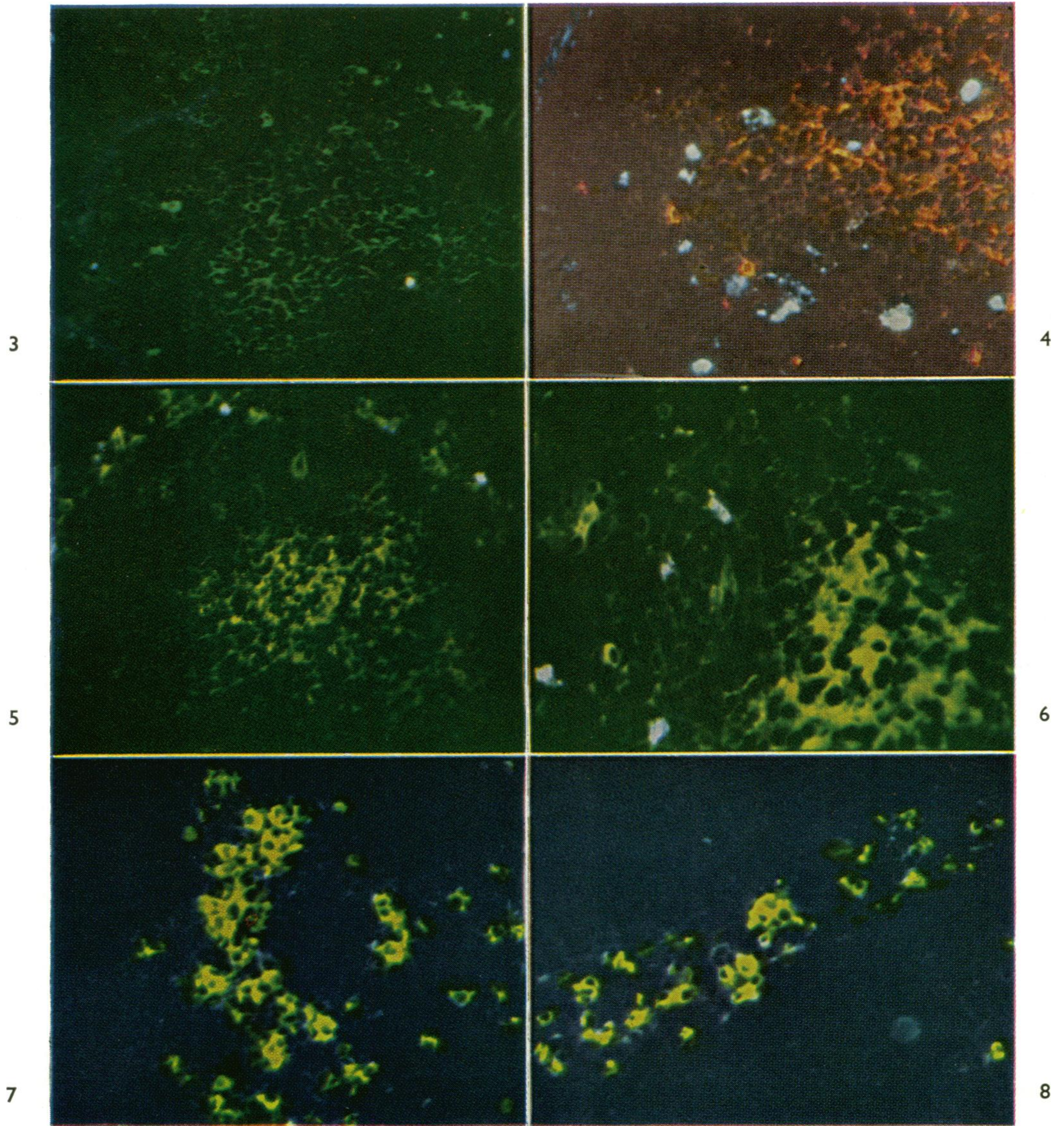
Fig. 4. Rabbit CG503-1. Germinal centre cells and plasma cells demonstrate orange-red fluorescence with anti-A5 rhodamine conjugate. ×300.

Fig. 5. Rabbit CG503-1. Germinal centre cells and plasma cells demonstrate yellow fluorescence when reacted in turn with anti-A4 used on the section in Fig. 3, and with anti-A5 used on the section in Fig. 4. The yellow fluorescence is the summation of the green and orange-red fluorescence. No cells show the fluorescence of the rhodamine conjugate alone. ×300.

Fig. 6. Rabbit CG503-1. Higher magnification of a germinal centre in a section reacted with anti-A4 fluorescein and anti-A5 rhodamine, as in Fig. 5. Homogeneous yellow fluorescence localized to the cytoplasm of cells grouped in close proximity can be seen. The nuclei show no fluorescence. ×480.

Fig. 7. Rabbit CG503-1. A group of plasma cells in a companion section from the same node as the foregoing sections. Yellow fluorescence in the cytoplasm of these cells is a result of reacting the section first with anti-A5 fluorescein conjugate, then with anti-A4 rhodamine. Control sections reacted with anti-A5 fluorescein were similar in staining to Fig. 3 and those reacted with anti-A4 rhodamine were similar to Fig. 4. ×480.

Fig. 8. Rabbit 20FZ-2. Medullary cord of plasma cells in a lymph node section reacted in turn with anti-A5 fluorescein and anti-A4 rhodamine. ×480.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same cells as the horse anti-RGG-Rh. Since the horse anti-RGG-Rh reacts with both the A4 and A5 γ-globulins, these results provide additional evidence that the A4 and A5 γ-globulins are produced in the same cell. The horse anti-RGG reacts also with the 10–20 per cent of γ-globulin molecules which have neither the A4 nor the A5 specificity. If it is assumed that these molecules are also produced in lymph node cells, these experiments indicate that γ-globulin molecules with neither A4 nor A5 are synthesized in the same cell with A4 and A5 γ-globulin molecules.

DISCUSSION

The finding that both A4 and A5 γ-globulin allotypic specificities are present within the same cell in lymph nodes of A4–A5 heterozygotes is based on experiments using an A5 anti-A4 γ-globulin and an A4 anti-A5 γ-globulin. Since there is a third allele at the *b* locus which determines an allotypic specificity A6, it would have been advantageous to prepare anti-A4 and anti-A5 in A6 homozygous rabbits. This would have simplified the technique since it would eliminate interaction of the A5 anti-A4 γ-globulin with the A4 anti-A5 γ-globulin and allow the simultaneous use of both conjugates on a single section. Furthermore it might have yielded further information on the intracellular sites of synthesis of the two allotypes. However, the allele for A6 is rare in rabbit populations studied thus far (Dray *et al.*, 1963a), and A6 homozygous rabbits were not available.

Although antibodies to γ-globulin allotypes can be produced by immunization with serum (Dray and Young, 1958), immune precipitates (Oudin, 1960a, b) or immune agglutinates (Dubiski *et al.*, 1959), DEAE–cellulose-prepared 7S γ-globulin was used for immunization in this study. This chromatographic procedure was used to avoid production of antibodies to rabbit serum proteins other than γ-globulin (Dray and Young, 1958).

However, an anti-allotype antiserum only identifies an allotypic specificity, presumably part of a specific polypeptide chain, which is present in 7S γ-globulin. The single arc characteristic of 7S γ-globulin on immunoelectrophoresis produced by each of these antisera does not rule out the presence of the same allotypic specificity on other proteins of fast and slow γ-mobility, since the lack of other precipitin arcs in this region can be a result of the relatively high concentration of 7S γ-globulin. Todd (1963) demonstrated that 19S γ-globulin reacted in ring tests with antibodies to γ-globulin allotypic specificities determined at the *a* and *b* loci. If it is assumed that the positive ring tests were not cross-reactions with polypeptide chains determined at other loci, then it would appear that the same polypeptide chains are components of both 7S and 19S γ-globulins. The presence of A4 and A5 allotypic specificities on 19S macroglobulins or proteins other than 7S γ-globulin does not influence the main question, i.e. whether the A4 and A5 allotypic specificities are produced in the same lymph node cell.

A further consideration in the specificity of the antisera is the finding (Dray and Nisonoff, 1963) that, although sheep anti-rabbit 7S γ-globulin precipitated 97 per cent of the 7S γ-globulin prepared from the sera of homozygotes and heterozygotes, only 80–90 per cent was precipitable by anti-A4 or anti-A5 or both antisera. Thus, 10–20 per cent of the 7S γ-globulin does not appear to be under control of alleles at the *b* locus. A γ-globulin allotypic specificity *P* has been described which appears to be determined at a third locus and to be present on a relatively small proportion of the molecules (Dray *et al.*, 1963a). Thus not only are the allotypic specificities determined at the *a* and *b* loci pro-

bably not limited to 7S γ-globulin, but not all of the 7S γ-globulin molecules carry the specificities determined at the *a* and *b* loci (Dray *et al.*, 1963b).

Despite these considerations of the specificity of the γ-globulin allotypes, the close correspondence of the cellular localization of γ-globulin with that of the allotypes in adjacent sections and the results of double staining with rhodamine-labelled horse anti-RGG demonstrate that the localization of the allotypic specificities A4 and A5 is equivalent to the localization of rabbit γ-globulin by the horse antiserum employed.

The cellular localization of the allotypic specificities of rabbit γ-globulin was the same as that found for human γ-globulin in lymph nodes of cancer patients by Ortega and Mellors (1957), using the fluorescent antibody method. These investigators found two major types of cells containing and therefore presumably synthesizing γ-globulin: plasma cells, both with and without Russell bodies, and the 'intrinsic cells' of the germinal centres. We found the same types of cells to contain the allotypes.

In this study, we have assumed that the presence of the allotypic specificities in the cytoplasm of plasma cells and intrinsic cells is equivalent to production of the allotypes by these cells.

The lymph nodes in these experiments were from unimmunized animals and contained fewer plasma cells than those studied by Ortega and Mellors. The concept of the germinal centres as 'immature organs of internal secretion of γ-globulin' is supported by our finding that in the rabbit lymph node sections the cells of the germinal centres were the most constant site of the γ-globulin allotypes. Normal nodes were studied in order to compare directly the findings on cellular localization with immunochemical studies of the same allotypes in the serum of unimmunized rabbits (Dray and Nisonoff, 1963; Dray *et al.*,

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Figs. 9–14. Photomicrographs of A4–A5 heterozygous lymph nodes were taken using dark field illumination with ultraviolet light at 360–370 mμ. Superanscochrome daylight type 35 mm film was employed with exposure times ranging from 1 to 7 minutes.

Fig. 9. Rabbit 20FZ-2. A selected medullary cord of plasma cells in a section reacted in turn with anti-A4 fluorescein and anti-A5 rhodamine. All fluorescent cells show the presence of both fluorescein and rhodamine, but the colour ratio of the two conjugates varies from cell to cell. ×480.

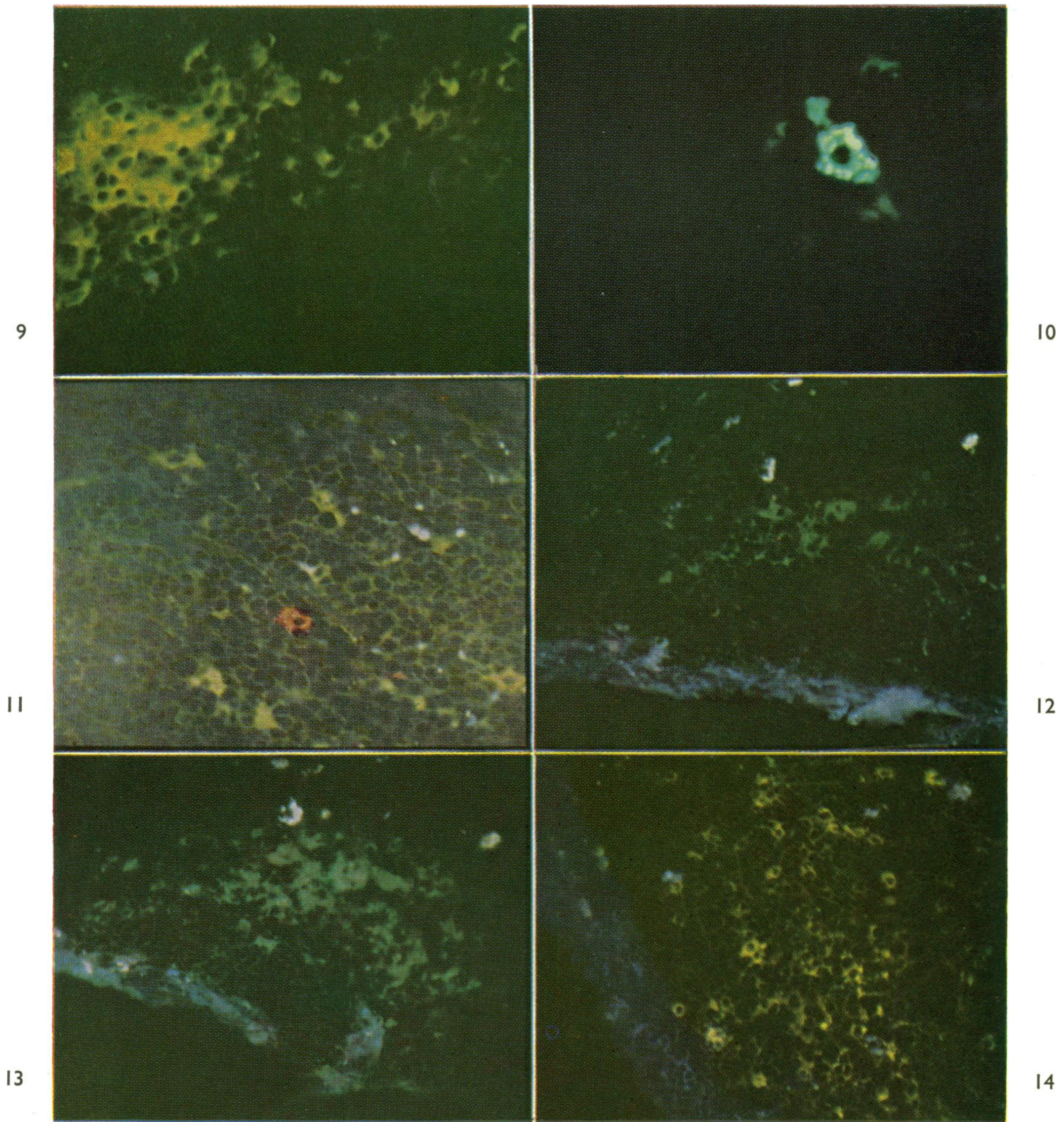
Fig. 10. Rabbit 20FZ-2. A rare Russell-body plasma cell stained with anti-A5 fluorescein conjugate and photographed 17 days later. Immature plasma cells are dimly visible. The blue fluorescence of what appears to be a small mature plasma cell is a result of the age of the tissue. ×720.

Fig. 11. Rabbit CG693-4. A section reacted in turn with anti-A4 fluorescein and anti-A5 rhodamine here demonstrates the very rare plasma cell which appears to be stained by the rhodamine conjugate alone. Its colour is in striking contrast to that of other cells in the field. ×480.

Fig. 12. Rabbit CG693-4. A section cut at an indicated 3 μ thickness reacted with anti-A5 fluorescein conjugate. A comparable field from the immediately adjacent section is shown in Fig. 13. Although differences are seen between the two sections, the white autofluorescent material and the blue autofluorescent tissue edge make cell-to-cell comparison possible if one section is rotated slightly. ×300.

Fig. 13. Rabbit CG693-4. An area directly comparable to Fig. 12, in the immediately adjacent section. This section has been reacted with anti-A4 fluorescein conjugate. Greater fluorescence is seen in this field than in Fig. 12 due in part to the greater amount of A4 than A5 in this heterozygote. Not all cells can be seen in both adjacent sections. One cell immediately below the central white autofluorescent matter can be easily identified and its cytoplasm is fluorescent in both sections, indicating that both allotypes are present within that cell. By careful matching and by photographic enlargement, over twenty other cells in these two fields can be identified and seen to have both allotypes in their cytoplasm. ×300.

Fig. 14. Rabbit CG693-4. A section reacted in turn with anti-A4 fluorescein conjugate and horse anti-rabbit γ-globulin rhodamine conjugate. The cytoplasm of cells demonstrates yellow fluorescence. No cells have rhodamine fluorescence alone. Similar results were obtained in sections first reacted with anti-A5 fluorescein conjugate, then with the horse anti-rabbit γ-globulin rhodamine conjugate. This indicates that all cells which contain γ-globulin as identified by horse anti-rabbit γ-globulin contain the allotypes A4 and A5. ×300.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1963b). A further consideration was the report of 'deletion' of the A5 allotypic specificity in antibody produced by one rabbit to a haptenic antigen (Gell and Kelus, 1962).

White (1958) used an immunofluorescent technique with conjugates of contrasting fluorochrome labels to investigate the localization of antibodies to two different antigens. In the medullary portion of the lymph nodes he found groups of plasma cells, some cells containing one antibody and some the other antibody. However, the cells of each germinal centre appeared to be making antibody to only one of the two antigens under study. Similarly Vazquez (1961) found spleen cells to have either one or the other antibody to two injected antigens; none of the cells had a mixture of colours. Coons (1959) studied formation of antibody to diphtheria toxoid and hen's egg albumin by staining alternate sections and found a marked difference in the sites of synthesis of the two antibodies in rabbits. In contrast to these studies of antibody formation in which two antibodies could be readily localized to different cells and groups of cells, no such differences in localization were evident in our study of two γ-globulin allotypes using similar methods.

In lymph node sections reacted with two conjugates of contrasting fluorochrome labels, there were a few cells which demonstrated a range of colour from near green to near red, suggesting that the cytoplasmic sites of synthesis of the two allotypes may differ. The lack of discrete areas of yellow and red fluorescence in most of the fluorescent cells may reflect close proximity of sites of synthesis, but this was not directly ascertainable since we did not have specific antibody to A4 without the A5 antigenic specificity and *vice versa.* Although this finding in just a few cells may represent an artefact of the complex double staining method used, it warrants further inquiry into specific sites of synthesis of each allotype in a heterozygous γ-globulin-producing cell.

The diffuse yellow fluorescence of the cytoplasm of most plasma cells and intrinsic cells, moreover, is not likely to be due to movement of γ-globulin during the preparation of the tissue sections since the fluorescence was always sharply localized to the cytoplasm leaving the nucleus a well-delineated dark circle.

Less than 1 per cent of the fluorescent cells in lymph node sections stained by the double conjugate method had only rhodamine fluorescence in their cytoplasm, and therefore appeared to be synthesizing just one of the allotypes, A4 or A5. These cells fluorescing orange-red alone could also occur as a result of technical factors. In the first step of this method, if antibody molecules were either poorly conjugated to fluorescein or had become dissociated from the label, they could still react with the appropriate allotype in the tissue section. This reaction would be poorly visible. In the second step, when the rhodamine conjugate reacted with the allotypic specificity of these antibody molecules, as well as with that of the γ-globulin in the cells of the section, only the red-orange fluorescence of rhodamine would be apparent.

However, if the presence of the rhodamine fluorescence alone in plasma cells truly reflects the status of production of the allotypes in these cells, then this finding in less than 1 per cent of fluorescent cells denotes either genetic mutation of the cell or the effect of external factors on protein synthesis. Since these cells apparently differ from nearly all the other γ-globulin-producing cells of the lymph nodes, one or the other mechanism must operate to inhibit the synthesis of one of the allotypes in these cells of the A4–A5 heterozygous rabbit.

Since practically all of the γ-globulin-producing cells contain both the A4 and A5 allotypes and since the ratio of A4 to A5 is roughly 2 : 1 in the serum of heterozygous rabbits, the allelic gene responsible for the production of A4 is presumably twice as efficient as the A5

gene. If the finding of rare cells which appear to be reacting with the rhodamine conjugate alone truly reflects allotype synthesis in these cells, then one would expect to find twice as many cells apparently producing A4 alone as those apparently producing A5 alone. However, this was not found. Rather, twice as many cells had only orange-red fluorescence in doubly stained sections where anti-A5 rhodamine conjugate was used in the second step as in those sections where anti-A4 rhodamine conjugate was used. This increases the probability that the rare appearance of rhodamine fluorescence alone in cells is the result of technical factors rather than of production of one allotype alone by these cells.

In either case, the results of these experiments support the conclusion that rabbit γ-globulin allotypes A4 and A5, determined by alleles at the *b* locus, in the heterozygous rabbit are produced in the same cell. In the double staining experiments in which horse anti-RGG rhodamine was used, an even smaller percentage of fluorescent cells was found in which the rhodamine conjugate was present without the fluorescein conjugate. These findings support the concept that the rare cells which showed the presence of the rhodamine conjugate without the presence of the first conjugate appeared as a result of the limitations of the technique. Since the horse anti-RGG precipitates 99 per cent of the rabbit 7S γ-globulins, the findings further indicate that all γ-globulin-producing cells in the lymph nodes examined synthesize both A4 and A5 γ-globulin molecules.

The present study together with the companion immunochemical studies (Dray and Nisonoff, 1963) deal with the same allelic genes and thus provide further information on the synthesis of γ-globulin.

Agar-gel tube analysis (Oudin, 1961) had shown that allotypic specificities Aa1 and Aa3, controlled by alleles at the *a* locus were not found on the same molecule. Dray and Nisonoff (1963) further showed that the allotypic specificities Ab4 and Ab5, determined by alleles at the *b* locus, were not present on the same molecules. The latter study also demonstrated in an Ab4–Ab4 homozygote, in an Ab5–Ab5 homozygote and in an Ab4–Ab5 heterozygote, that 10–20 per cent of the γ-globulin was not precipitable by anti-A4, anti-A5 or both antisera.

Dray *et al.* (1963b) also demonstrated by the same method that in an Aa1–Aa3, Ab4–Ab5 double heterozygous rabbit, there exists Aa1–Ab5 and Aa1–Ab4 7S γ-globulin molecules. They postulated that Aa3 is similarly associated with Ab4 and Ab5 so that in this rabbit heterozygous at both the *a* and *b* loci, there were γ-globulins with four different combinations of specificities: Aa1–Ab4, Aa1–Ab5, Aa3–Ab4 and Aa3–Ab5. Theoretically, these four allotypes of γ-globulin could be synthesized separately in four populations of cells. The present study with the fluorescent antibody method has found that in the heterozygous rabbit, both Ab4 and Ab5 are produced in the same cell. This rules out the possibility of there being four cell populations. By analogy, it is reasonable to expect that the allelically controlled allotypic specificities at the *a* locus are likewise produced within the same cell and that in an Aa1–Aa3, Ab4–Ab5 double heterozygote, all four molecules are produced within the same cell. The localization of allotypes determined at the *a* locus is under investigation to test this hypothesis.

The information presently available concerning the synthesis of γ-globulin derived from studies of its allotypic specificities determined at the *a* and *b* loci parallels to a remarkable degree the synthesis of human haemoglobin derived from studies of its α and β chains determined by alleles at two independent loci (Singer and Itano, 1959; Itano and Robinson, 1960). The reason allelic genes do not contribute to the same molecule although both alleles contribute to the synthesis of γ-globulin within the same cells may lie in the follow-

ing concept. Polypeptide chains determined by one allele form pairs immediately after biosynthesis and such pairs do not combine with similarly formed pairs determined by the allelic gene but do combine with pairs of polypeptide chains determined at other loci (Itano, 1957; Singer and Itano, 1959; Itano and Robinson, 1960; Dray and Nisonoff, 1963; Dray *et al.*, 1963b).

The relative concentration of the products of allelic genes found in serum may shift considerably from normal as a result of 'suppression' of one product by passively administered antiserum *in utero* (Dray, 1962) and by immunization (Gell and Kelus, 1962; Reider and Oudin, 1963; Lark, Eisen and Dray, unpublished data). For the analogous Gm groups in man, the concentration of allelic products may also change as a result of immunization (Mårtensson, 1962) and also in pathology (Mårtensson, 1961, 1962, 1963; Harboe, Osterland, Mannik and Kunkel, 1962).

It has been argued (Harboe *et al.*, 1962; Mårtensson, 1963) that the normal plasma cell in a heterozygous individual has a restricted capacity to express genetic information in its protein product, i.e. only one allele at a locus is active. Contrary to these arguments which stem from studies of myeloma proteins, the present investigation of cellular production of normal 7S γ-globulin in the rabbit has shown that the normal plasma cell in a heterozygote has the ability to produce both allelic products. Thus the observed changes in concentration of allelic products found in serum may not be a result of a change in the ratio of cells producing each allelic product but rather may be a result of a change in the relative quantities of the two products synthesized within cells capable of synthesizing both products.

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