RABBIT LYMPHOID CELLS DIFFERENTIATED WITH RESPECT TO α-, γ-, AND μ- HEAVY POLYPEPTIDE CHAINS AND TO ALLOTYPIC MARKERS Aa1 AND Aa2*

By J. J. CEBRA,‡ Ph.D., J. E. COLBERG,§ M.D., and S. DRAY,‖ M.D.

(From the Department of Microbiology, University of Florida, Gainesville, and the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland)

Plates 66 to 69

(Received for publication 1 October 1965)

Immunoglobulins of the γA-, γG-, and γM-classes are distinguished by their heavy polypeptide chains, called α, γ, and μ respectively, and are related by their common light polypeptide chains (1, 2). Staining of human lymphoid tissue with fluorescent antibody reagents (3) specific for particular polypeptide chains has revealed a striking differentiation of fluorescing cells with respect to their content of the different classes of heavy (4–7) and types of light (7–9) immunoglobulin chains. Recent technical improvements in fluorescent antibody procedures have facilitated simultaneous staining with mixtures of fluorescein- and rhodamine-labeled antibodies and the enumeration of lymphoid cells containing each of any two immunoglobulin polypeptide chains (10–13, 7, 8). When rabbit lymphoid tissue was stained with antilight chain and anti-γ-chain conjugated with contrasting fluorochromes, 63 to 78% of all fluorescing cells were found to contain both component chains of γG-immunoglobulin in detectable amounts (13). The balance of these cells stained with antilight chain alone and presumably contained immunoglobulins of a class other than γG.

Further characterization of the rabbit immunoglobulins, including the isolation of γA-immunoglobulin (14) and of γM-antibody (15), has led to the preparation of specific fluorescent antibody reagents which react with either the α, γ, or μ heavy polypeptide chains or with either of two genetically controlled antigenic markers present on heavy chain, Aa1 and Aa2 (16–19). This comprehensive report of the cellular distribution of various rabbit immunoglobulin heavy chains indicates a remarkable differentiation of most fluorescing

* Supported by Grant AI-05042 from the National Institute of Allergy and Infectious Diseases, Bethesda.

‡ Recipient of a Public Health Research Career Development Award (K3-AI-11,469).

§ Present address: Department of Surgery, University Hospitals, Cleveland.

‖ Present address: Department of Microbiology, University of Illinois at the Medical Center, Chicago.

lymphoid cells with respect to the class or allotype of heavy chain detectable in their cytoplasm.

Materials and Methods

Fluorescent Antibody Reagents.—Anti-μ-chain was prepared by immunizing goats with purified anti-salmonella γM-antibody (15); anti-α-chain, by immunization with homogeneous γA-immunoglobulin isolated from rabbit colostrum (14). Bis-diazobenzidine cross-linked insoluble rabbit γG-immunoglobulin was used to absorb the small amount of nonclass specific antibody present in these reagents (7). Preparation of goat anti-γ-chains has been described (20). Anti-Aa1 and anti-Aa2 antisera were prepared by immunization of Aa^3/Aa^3 homozygous rabbits with γG-immunoglobulin isolated from Aa^1/Aa^1 and Aa^2/Aa^2 homozygous rabbits (16, 17). Fluorescent reagent immunoglobulins which gave bright specific staining at high dilution were prepared by conjugation with fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate as detailed previously (12, 13).

Fluorescent anti-Aa1 reagents did not stain cells in lymphoid tissue from Aa^2/Aa^2 rabbits and, conversely, fluorescent anti-Aa2 reagents did not stain cells in tissue from Aa^1/Aa^1 rabbits.

Pairs of conjugated antibody reagents with contrasting fluorochromes were prepared by mixing the individual reagents to achieve optimal dilutions of each (13). The final γG-immunoglobulin concentration of each fluorescent antibody reagent and the ratio of its optical densities at 280 and 515 mμ for rhodamine (red) conjugates and at 280 and 495 mμ for fluorescein (green) conjugates was as follows: red anti-γ (0.25 mg/ml) 2.1:1; red anti-α (0.45 mg/ml) 4.5:1; green anti-α (0.22 mg/ml) 4.3:1; green anti-μ (0.34) 7.3:1; red anti-Aa2 (0.61 mg/ml) 9.4:1; and green anti-Aa1 (0.61 mg/ml) 3.7:1.

Tissue Preparations.—Lymphoid tissue was obtained from normal rabbits or rabbits which had been hyperimmunized with either dinitrophenylated (DNP) horse ferritin (5 mg), administered in complete Freund's adjuvant at multiple sites (7), or with *Salmonella typhimurium* as described (15). All lymphoid tissue donor rabbits except DNP-ferritin 2 (Aa^1/Aa^1) were selected for Aa^1/Aa^2 heterozygosity from a large group of rabbits and they included New Zealand Whites, Flemish, and California Whites. Tissue touch prints and tissue sections (3 to 6 μ) made in a cryostat were obtained from spleen and lymph nodes taken from anti-DNP ferritin animals 2 to 3 wk after the date of injections and from anti-salmonella animals in a 2 month period after the prolonged course of immunization. The same pair of reagents was used to stain two sets of spleen imprints made from different parts of the same organ. Stained specimens were examined under a Leitz ultraviolet microscope with a Corning 5840 exciting filter and K2, 23A, and 57A eyepiece filters (13). From 100 to 500 positive cells were counted for each preparation and the relative percentage of cells stained by each or both of the reagents was calculated and is given in Tables I and II.

RESULTS

Cellular Localization of Heavy Chains of Different Classes.—The results of experiments designed to determine the distribution of the α-, γ-, and μ- heavy chains among immunoglobulin-containing cells are summarized in Table I. The principle observation is that the class specific heavy chains of rabbit immunoglobulins are localized and presumably synthesized in separate cells. At most 2%, and usually far fewer, of the cells appeared to stain with both reagents of any particular pair. This observation was the same whether one considered the cells of spleen imprints (Figs. 1 to 3) or the cells in sections of very active popliteal lymph nodes (Figs. 10 to 13). These latter contained many

TABLE I

Distribution of α-, μ-, and γ- Heavy Polypeptide Chains Among Rabbit Lymphoid Cells

Donor rabbit	Relative No. of cells stained by each or both of the following pairs of fluorescent antibody reagents											
	Green anti-α + red anti-γ				Green anti-μ + red anti-γ				Red anti-α + green anti-μ			
	Tissue*	α	γ	α + γ	Tissue*	μ	γ	μ + γ	Tissue*	α	μ	α + μ
		%	%	%		%	%	%		%	%	%
Anti-Salmonella 1	spl—P	5	95	—	spl—P	13	87	—	spl—P	29	71	—
	spl—P	6	94	—	spl—P	16	83	1	spl—P	30	70	—
Anti-Salmonella 2	Not done				spl—P	24	75	1	Not done			
					spl—P	15	84	1				
Anti-Salmonella 3	pln—S	4	96	—	spl—S	47	53	—	spl—S	25	75	—
	pln—S	3	97	—	mln—S	29	71	—	pln—S	7	93	—
					pln—S	64	36	—	pln—S	3	97	—
					pln—S	65	35	—				
Anti-DNP-Ferritin 1	spl—P	7	93	—	spl—P	25	75	—	spl—P	22	78	—
	spl—P	10	90	—	spl—P	32	68	—	spl—P	23	77	—
Anti-DNP-Ferritin 2	spl—P	15	85	—	spl—P	23	77	—	spl—P	22	78	—
					spl—P	22	77	1	pln—P	18	81	1
									pln—P	21	77	2
									spl—P	17	83	—

* Type of tissue preparation: spl, spleen; pln, popliteal lymph node; mln, mesenteric lymph node; P, imprints; and S, cryostat sections.

active germinal centers and a profusion of fluorescing cells. Great clusters of fluorescing cells in lymph node sections or imprints were observed and often cells stained with one or the other fluorescent reagent predominated in these areas. However, these regions always contained at least a small minority of cells stained only with the contrasting reagent and these latter were strewn throughout the clusters in apparent random fashion. Figs. 1 to 3 and Figs. 10 to 13 show the use of the filters 23A and 57A to distinguish between the differentially stained cells after the application of the pair of reagents red anti-γ-chain and green anti-μ-chain.

Cellular Localization of Allotypic Markers Aa1 and Aa2.—To determine whether lymphoid cell differentiation with respect to variety of heavy chain produced also extended to heavy chains differentiable on the basis of allotypic markers we examined rabbits heterozygous at the *a* locus (Aa^1/Aa^2). The allotypic markers controlled by the *a* locus have been located on the γ-chain (18, 19). It is not clear whether this set of antigenic sites can be present on all classes of heavy chain, as has been suggested by some reports (21, 22). Table II gives the distribution of the two heavy chain allotypic markers Aa1 and Aa2 among the lymphoid cells of six rabbits heterozygous at the *a* locus. The results indicate that the two markers are essentially confined to different cells in the spleen. A small fraction of the fluorescing cells from lymph node imprints appeared to double stain. However, the lymph node imprints from anti–DNP-ferritin rabbit 1 showed a high fluorescing background of extracellular globulin and designation of cells as being clearly double stained was difficult.

Figs. 4 to 6 show the separate localization of the allotypic markers in cells of spleen and Figs. 7 to 9 show a section of popliteal lymph node in which cells can be differentiated on the basis of their content of either Aa1 or Aa2 antigenic sites. The ratio of cells containing the Aa1 marker to those containing the Aa2 marker varied somewhat with individual rabbits and with the lymphoid tissue examined. Usually, the proportion of fluorescing cells containing the Aa1 marker predominated over those containing the Aa2 marker; the range was 53 to 86% Aa1 cells to 14 to 47% Aa2 cells.

The rabbit intestine, rich in lymphoid tissue, has been found to contain cells which stain separately for one or another class of heavy chain (23). Double staining of several cross-sections of intestines from the rabbits listed in Table II as anti-salmonella 3 and "nonimmunized" with red anti-Aa2 and green anti-Aa1 likewise showed clear separate cellular localization of the two allotypic markers. Out of a total of 655 fluorescing cells counted for anti-salmonella 3 tissue, and 405 cells counted for the tissue of the nonimmunized heterozygote, 50 and 46% respectively contained the Aa1 marker while the balance of the stained cells contained the Aa2 marker.

TABLE II

Distribution of Aa1 and Aa2 Allotypic Genetic Markers of Heavy Polypeptide Chains Among Lymphoid Cells of Aa^1/Aa^2 Heterozygous Rabbits

Donor rabbit	Relative No. of cells stained by green anti-Aa1 and red anti-Aa2			
	Tissue*	Aa1	Aa2	Aa1 + Aa2
		%	%	%
Anti-Salmonella 1	spl—P	81	19	—
	spl—P	79	21	—
	spl—P	79	21	—
	spl—P	86	14	—
	pln—P	85	15	—
	pln—P	88	12	—
Anti-Salmonella 2	spl—P	79	21	—
	spl—P	85	15	—
	spl—P	77	23	—
Anti-Salmonella 3	spl—P	75	25	—
	spl—S	78	22	—
	spl—S	69	31	—
	pln—P	60	40	—
	pln—S	53	47	—
	pln—S	54	46	—
	mln—S	69	31	—
Anti-DNP-Ferritin 1	spl—P	72	28	—
	spl—P	72	27	1
	spl—P	65	34	1
	spl—P	71	28	1
	mln—P	61	33	6
	mln—P	62	29	9
	mln—P	58	20	22
Anti-DNP-Ferritin 3	spl—P	81	19	—
	spl—P	86	14	—
	pln—S	81	17	2
	pln—S	69	30	1
	pln—S	76	24	—
Nonimmunized	spl—P	86	14	—
	pln—S	68	32	—
	pln—S	71	29	—

* Type of tissue preparation: spl, spleen; pln, popliteal lymph node; mln, mesenteric ylmph node; P, imprints; and S, cryostat sections.

DISCUSSION

Our present findings indicate that the majority of fluorescing lymphoid cells were differentiable on the basis of their content of one or another class of heavy chain. The presence of this kind of cellular differentiation is in accord with observations made with human lymphoid tissue (4–9). The additional significance of our data, obtained using the rabbit, is that the relative frequencies can be calculated for all three classes of heavy chains in cells from the same tissue of an individual animal. The values for the cellular distribution of the three classes of heavy chain were calculated for each rabbit from the experimentally determined ratios of singly stained cells in tissues stained for two classes of heavy chains; i.e. α:γ, μ:γ, and α:μ (Table I). The α:μ ratio could also be derived by calculation from the α:γ and μ:γ ratios and these calculated values were in good agreement with the α:μ ratio determined directly. The average of the calculated and directly determined α:μ values were employed to correct the α:γ and μ:γ ratios used in the calculation of the relative number of cells containing either α-, μ- or γ-chains. The per cent of cells containing either α-, μ-, or γ- heavy chains was in the range of 5 to 8%, 14 to 21%, and 71 to 81% respectively for all animals except for anti-salmonella 3 (Table I). The data obtained for a draining popliteal lymph node of the anti-salmonella rabbit 3 suggest a shift in relative proportions of cells in favor of those containing μ chain compared with the distribution of fluorescing cells found in the spleen and mesenteric lymph node of the same animal.

Previously we have found that 23 to 37% of those spleen cells occurring in nonimmunized or immune rabbits which stained for light chain did not contain the γ-chain (13). Our present data would indeed indicate that these cells contained either μ- or α-chain and were presumably synthesizing γM- or γA-immunoglobulin. It is apparent that only a low percentage (5 to 8%) of the fluorescing cells in spleen or in lymph nodes contained γA-immunoglobulin. The few cells potentially synthesizing γA-immunoglobulin accords well with the low concentration of this immunoglobulin (180 μg/ml) found in adult rabbit serum (14). The low percentage of cells containing γA-immunoglobulin (5 to 8%) and the low contribution of γA-immunoglobulin to the total serum immunoglobulins ($\sim$1.5%) in the rabbit contrasts with the corresponding values found in the human of 28 to 50% for γA-immunoglobulin containing cells (7) and 17% for the γA-immunoglobulin in serum (24).

The relative proportion of immunoglobulin-containing cells which stained for the μ-chain alone was about 14 to 21% in both spleens and lymph nodes of all animals except anti-salmonella rabbit 3 (Table I). The contribution of γM-immunoglobulin to the total rabbit serum immunoglobulins is not known. However, the proportion of γM-antibody specific for the DNP-group and precipitable with DNP-serum albumin was determined to be 6% for the serum of

anti-DNP-ferritin rabbit 2. All precipitable antibody was dissociated from antigen with ε-DNP-lysine, separated from antigen on DEAE-Sephadex, and analyzed by analytic ultracentrifugation and Sephadex G-200 gel filtration.

A rough correlation can thus be made between the proportion of cells apparently synthesizing a given immunoglobulin and the proportion of that same immunoglobulin in serum. Obviously other factors, such as differential ability of globulins to permeate the extravascular spaces or to become tissue bound and differential turnover rates of the various immunoglobulins also would affect their relative concentrations in serum.

The data presented in Table II indicate that lymphoid cell differentiation in hyperimmune rabbits also exists to the point of separate localization and presumably separate synthesis of different allotypes of heavy chains. A similar kind of differentiation has been inferred for human cells by Harboe et al. from the particular genetically controlled markers present on various myeloma proteins (25). In general, the myeloma proteins found in patients who were heterozygous at the *Gm* or *Inv* locus contained only one of the two markers believed to be controlled by a given locus and hence it was reasoned that the plasma cells producing these proteins were expressing only one of their two allelic cistrons.

Differential counts of fluorescing cells present in the spleen and lymph nodes of six Aa^1/Aa^2 heterozygous rabbits (Table II) indicated that cells containing the Aa1 marker outnumbered those containing the Aa2 markers by about 3 to 1. A similar count made on a single rabbit, reported recently by Pernis et al. (26), revealed a similar imbalance of cells in favor of those containing the Aa1 marker, which was detected separately in 62% of the fluorescing cells. The relative serum concentrations of immunoglobulin molecules bearing the Aa1 marker vs. the Aa2 marker has been found to be about 85:15 for a group of Aa^1/Aa^2 heterozygous rabbits (27). Dr. Rose Mage has likewise determined the relative serum concentrations of Aa1 vs. Aa2 molecules present in the sera taken during exsanguination of the "anti-DNP-ferritin 3" and "nonimmunized" rabbits (Table II) and found these ratios to be 80:20 and 90:10 respectively. Thus there seems to exist a fairly close relationship between the relative number of cells apparently synthesizing an immunoglobulin molecule of a given allotype and the relative serum concentration of molecules bearing a given allotypic marker. The practicality of making reliable differential lymphoid cell counts after double fluorescent staining was shown initially after localization of κ- and λ-light chains in human lymphoid tissue (7, 8). A good correlation was found between the relative numbers of cells containing κ- vs. λ-chain (63:36) and the relative serum concentrations of molecules bearing κ- vs. λ-chains. There is also some indication that the relative numbers of cells synthesizing each of two allotypes of rabbit light chains, Ab4 and Ab5, may

be correlated with the relative serum concentrations of the corresponding molecules (26). In all cases where there appears to be a good correlation between cells containing differentiable immunoglobulins and serum concentrations of the corresponding molecules, it is probable that the pertinent comparisons, with respect to type or allotype, are being made within a given class of immunoglobulin or class by class. Thus differences in half-life and rates of passage to extravascular spaces of the different classes of immunoglobulins do not disrupt the apparent correlations between number of synthesizing cells of a particular kind and the serum concentration of their product.

That most rabbit lymphoid cells were differentiable on the basis of their content of only one or the other product of two allelic cistrons has recently been independently reported by Pernis et al. (26). This cellular differentiation was found to exist with respect to allotypic markers of light chains controlled by the *b* locus as well as with respect to the products of the *a* locus discussed above. However, in an earlier study of mesenteric lymph nodes from nonimmunized Ab^4/Ab^5 heterozygotes, less than 1% of the fluorescent cells were found to contain one allotype without the other (28). The experimental design of this latter light chain study differed from that used in the present investigation in the following ways: (*a*) mesenteric lymph nodes from nonimmunized rabbits were the primary object of study and consequently most fluorescing cells were present in the follicles rather than in the medullary cords; and (*b*) sequential staining was employed (e.g. green anti-Ab4 followed by red anti-Ab5) because the reagents reacted with each other. It seems possible that a pair of mutually reacting fluorescent antibody reagents, even when applied sequentially, may result in double staining of all cells containing either or both of two antigens being sought. This apparent double staining could come about by elution of some of the first reagent from the tissue during staining with the second and subsequent fixation of complexes containing both fluorochromes to cells containing either allotypic specificity. Another explanation for the variance between the present observations and those of Pernis et al. (26) on one hand and those of Colberg and Dray (28) on the other could be that the latter observations were indeed mainly directed towards germinal centers, which contain cells not easily differentiable with respect to content of immunoglobulin antigenic markers. Lymph nodes from human patients have been reported to contain cells located in germinal centers which stained for both of either two types of light chains or two classes of heavy chains, although most cells in spleen and some cells in nodes were described as being differentiated with respect to class and type of immunoglobulin (5, 9).

Thus, lymphoid cell differentiation with respect to polypeptide chain content can certainly occur. This type of cellular differentiation represents either the qualitative presence or absence of particular polypeptide chains or a marked

imbalance in the amounts of the various classes and types of chains in individual cells. The limit of sensitivity of fluorescent antibody reagent prevents clear resolution of these possibilities. Likewise the conditions under which individual cells might synthesize more than one class of polypeptide chain or two allelic chains requires further investigation.

Among the many pertinent experiments that would be helpful on these points would be: (*a*) use of "cloned" lymphoid cells to determine how the producers of the various chains arise; (*b*) study of the development of globulin-containing cells after initial antigenic stimulation to detect a possible transition of cells from production of μ-chain to synthesis of γ-chain as suggested by Nossal et al. (29); and (*c*) determination of the influence of particular antigens on the cellular distribution of the classes of antibody synthesized in the response.

SUMMARY

Lymphoid cells present in spleen and lymph nodes of hyperimmune rabbits were found to be differentiated with respect to the class of immunoglobulin heavy chain which they contained. The relative proportions of cells containing the various heavy chains were as follows: α-chain (5 to 8%), μ-chain (14 to 21%), and γ-chain (71 to 81%). The allotypic markers Aa1 and Aa2, found on heavy chains, were also found to be separately localized in cells of Aa^1/Aa^2 heterozygous rabbits. The ratio of cells in spleen and lymph nodes containing the Aa1 marker to those containing the Aa2 marker varied with individual rabbits; the range was 53 to 88% Aa1 versus 12 to 47% Aa2.

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EXPLANATION OF PLATES

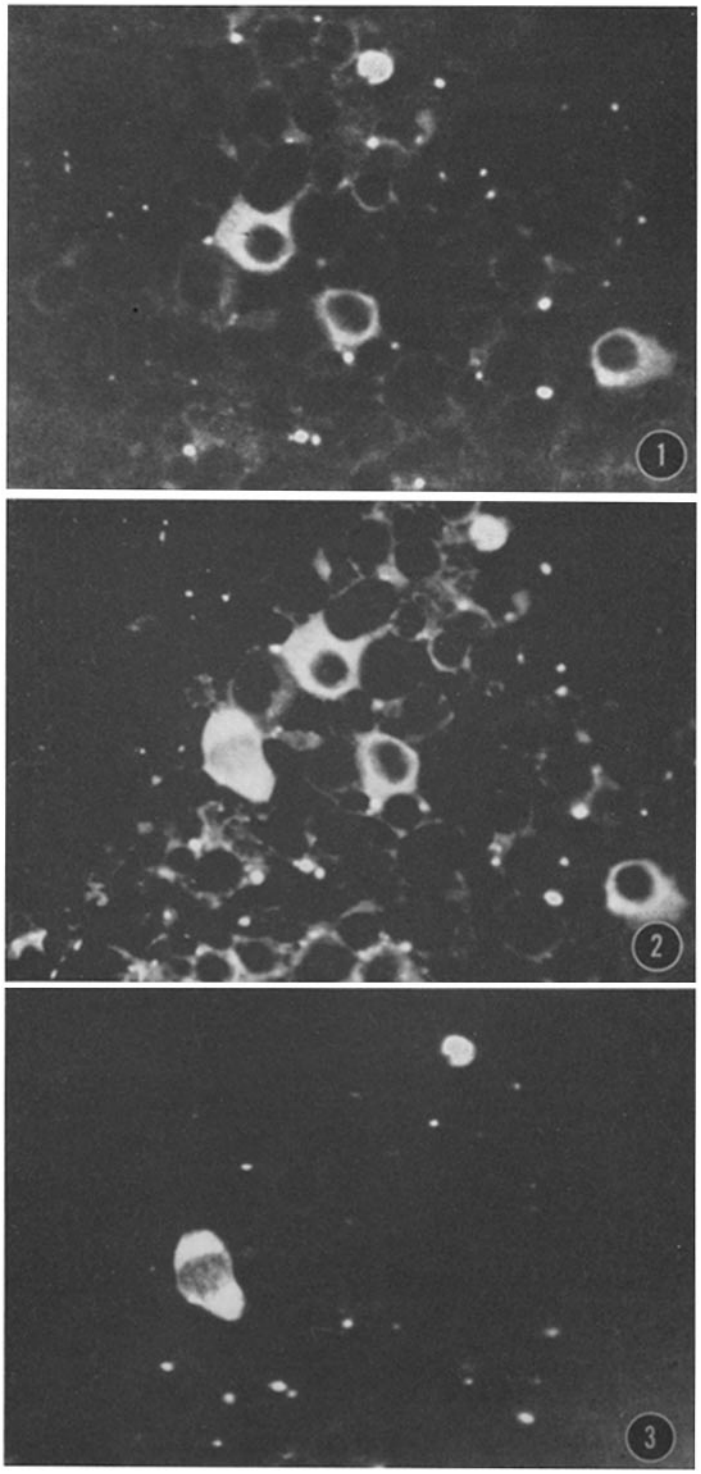
FIGS. 1 to 13. Figs. 1 to 6 are photomicrographs of cells present in spleen imprints from hyperimmune rabbits (Aa^1/Aa^2 heterozygotes). Figs. 7 to 13 are photomicrographs of cryostat sections of the popliteal lymph nodes of the same hyperimmune rabbits. The immunofluorescence was originally photographed using Super-Anscochrome, ASA200, and the 54 × oil immersion objective. Whitish autofluorescent aggregates, present in stained or unstained tissue preparations, can be seen through all filters (K2, 23A, and 57A). Figs. 1 to 9, × 650; Figs. 10 to 13, × 400.

PLATE 66

FIG. 1. Same field shown in Figs. 2 and 3 taken through the red barrier filter (23A) showing the cells stained with red anti-γ-chain.

FIG. 2. A field showing four cells stained with the mixture of reagents; red anti-γ-chain and green anti-μ-chain taken through the neutral (K2) filter.

FIG. 3. Same field shown in Figs. 1 and 2 taken through the green window filter (57A) showing the single cell stained with green anti-μ-chain.



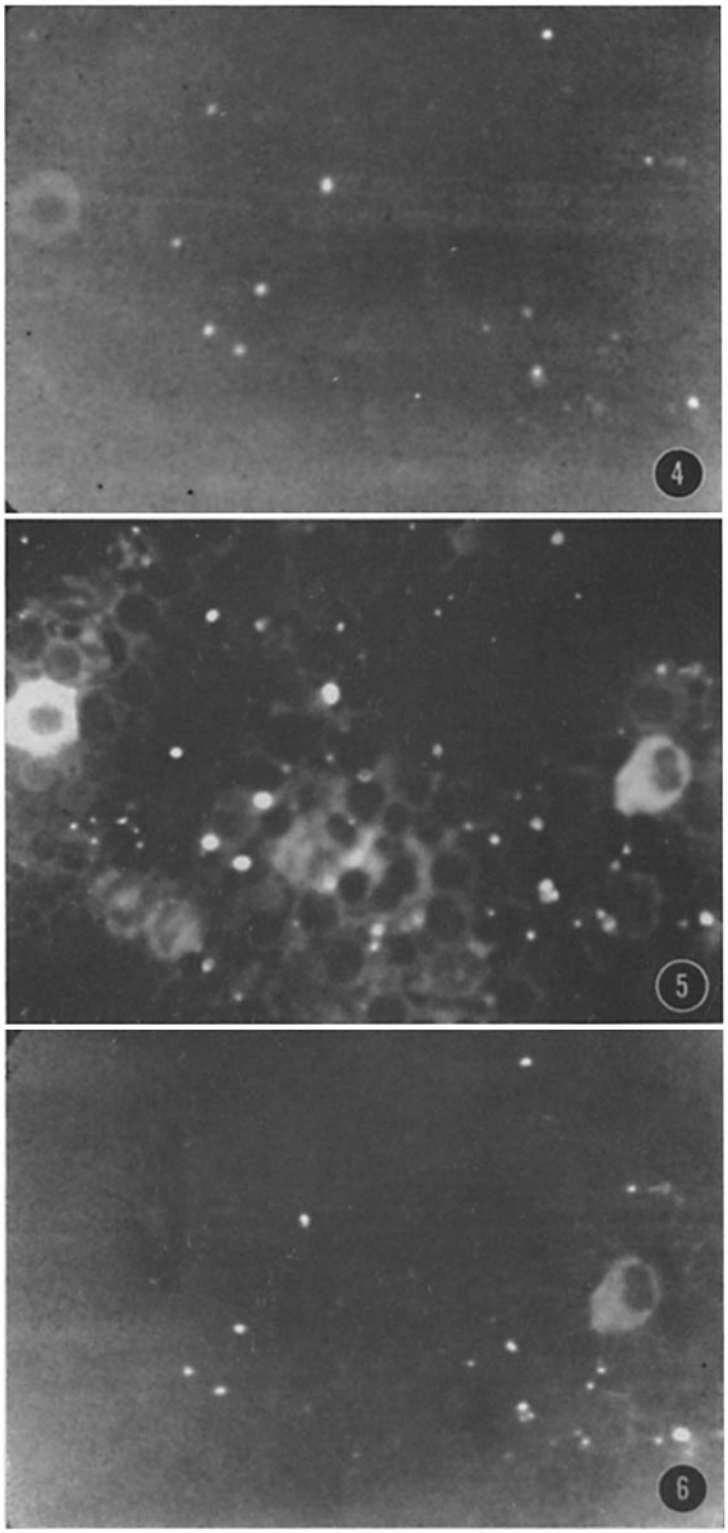
(Cebra et al.: Differentiated lymphoid cells)

PLATE 67

FIG. 4. Same field shown in Figs. 5 and 6 taken through the red filter (23A) showing the cell stained only by red anti-Aa2.

FIG. 5. A field showing two fluorescing cells, one stained only by the green anti-Aa1 reagent (right), and the other stained only by the red anti-Aa2 reagent (left).

FIG. 6. Same field shown in Figs. 4 and 5 taken through a green filter (57A) showing the cell stained only by the green anti-Aa1.



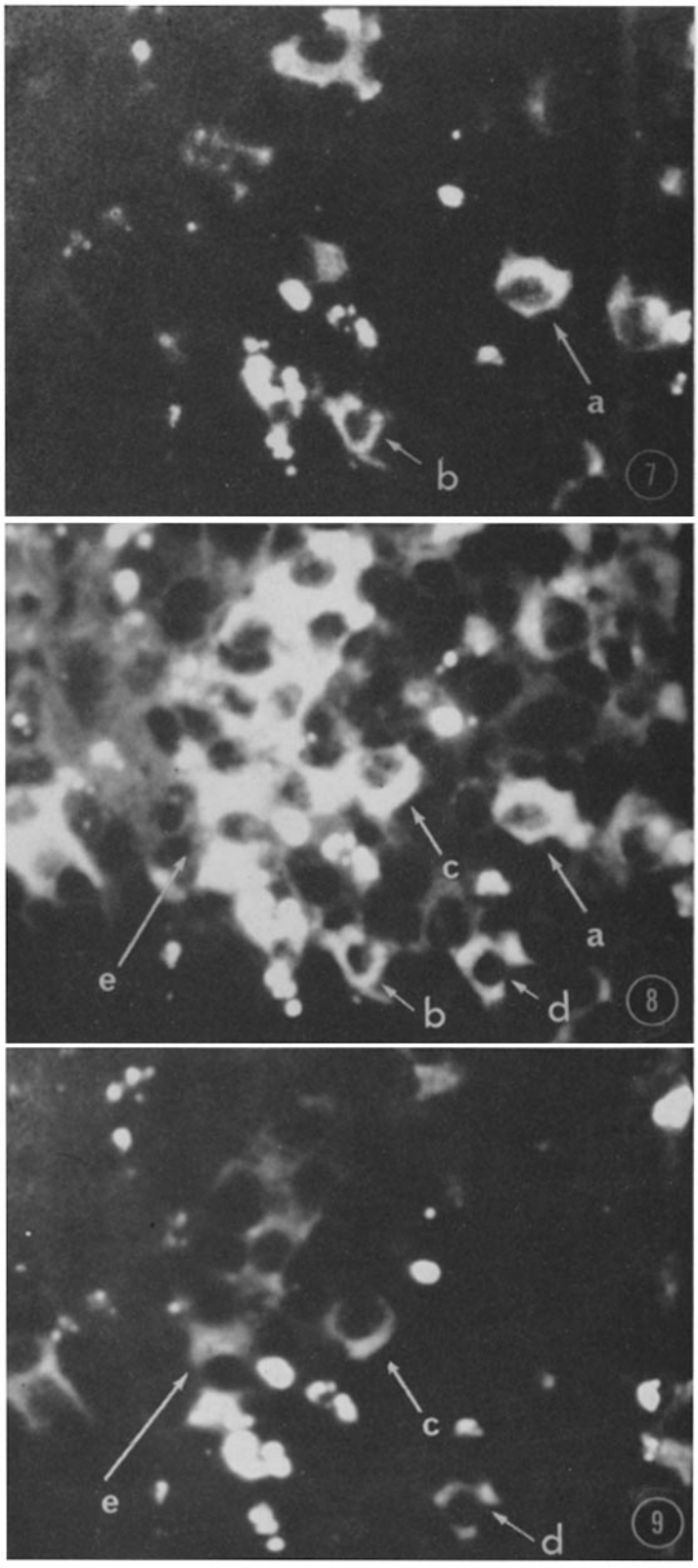
(Cebra et al.: Differentiated lymphoid cells)

PLATE 68

FIG. 7. Same field as shown in Figs. 8 and 9, but taken through the red filter (23A). Two conspicuous red cells, stained with red anti-Aa1 and marked *a* and *b*, can be seen in this field and also in Fig. 8, taken through the K2 filter. These cells are not apparent in the same field taken through the green filter and shown in Fig. 9.

FIG. 8. A section of the popliteal lymph node of an Aa^1/Aa^2 heterozygous rabbit stained with green anti-Aa1 and red anti-Aa2. Five cells are marked as a guide to Figs. 7 and 9 observed using the K2 filter. Two of these cells fluoresce red and are marked *a* and *b*. The other three fluoresce green and are marked *c*, *d*, and *e*.

FIG. 9. Same field as shown in Figs. 7 and 8, but taken through the green filter (57A). Three conspicuous green cells, stained with green anti-Aa1 and marked *c*, *d* and *e*, can be seen in this field and also in Fig. 8, taken through the K2 filter. These cells are not apparent in the same field taken through the red filter and shown in Fig. 7.



(Cebra et al.: Differentiated lymphoid cells)

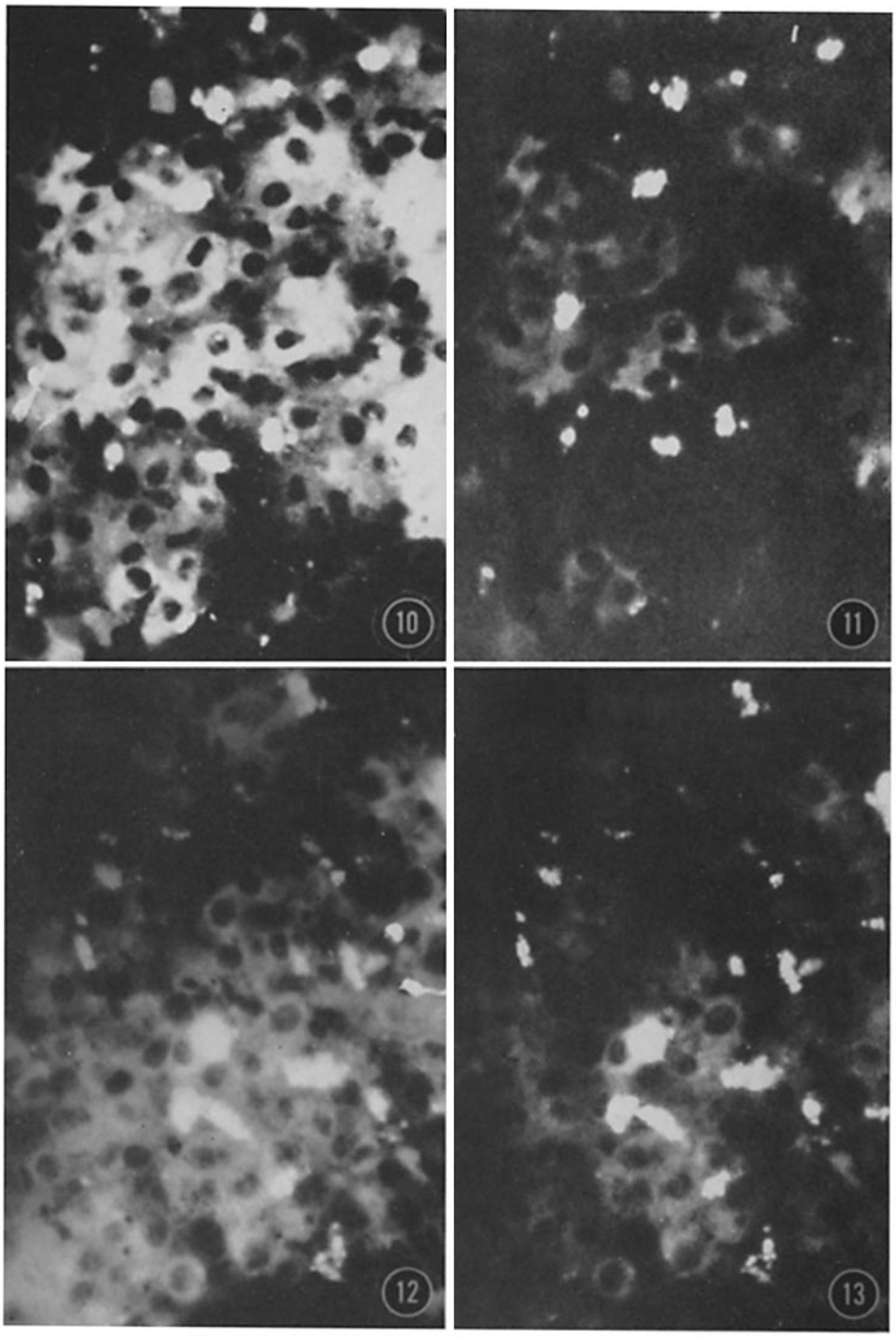
PLATE 69

FIG. 10. A section of a popliteal lymph node stained with green anti–μ-chain and red anti–γ-chain. The picture was taken using the neutral (K2) filter.

FIG. 11. The same field as shown in Fig. 10, but taken through the green filter. Only the cells fluorescing green and containing μ-chain are apparent. These were interspersed among the cells fluorescing red, which are not visible here but apparent in Fig. 10.

FIG. 12. Another section of a popliteal lymph node stained with green anti–μ-chain and red anti–γ-chain and observed through the neutral (K2) filter.

FIG. 13. The same field shown in Fig. 12, but taken through the red filter. Only the cells fluorescing red and containing γ-chain are apparent. Again, these cells were intermingled with the green fluorescing cells, nearly invisible here but clearly apparent in Fig. 12.



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