CRITERIA OF THE AGE OF LYMPHOCYTES IN THE PERIPHERAL BLOOD

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Unlike most of the other cells in the adult body, all those found normally within the circulating blood continue throughout life to develop from primitive forms. In any analysis of the blood in disease, it is of the greatest importance to be able to determine the state of maturity of the cellular elements. The present work is concerned with criteria of the age of the lymphocyte.

There are two principal sites of origin for the blood cells: the bone marrow and the lymph nodes. Most of our present knowledge concerning the maturation cycle and the relative age or youth of cells in the circulation has been derived from studies of the erythrocytes and granulocytes arising in the bone marrow. By noting variations in certain cytoplasmic (1, 2) or nuclear (3–6) criteria, the relative age of these cells can be estimated and valuable information be obtained. The studies thus far recorded of lymphocytes and lymphatic tissue have not been of a character to contribute materially to the establishment of acceptable criteria of maturation for this strain of white blood cells. A search of the literature reveals some attempts to link the so called lymphocyte with the other blood cells as a hemoblast or precursor in the development of the various well recognized definitive types. Arneth (7), following his work on the neutrophilic granulocyte, published an index of nuclear variation in the lymphocytes to which he attached maturative significance. His studies have not received the verification and acceptance accorded to his observations on the polymorphonuclear leucocyte.

There are several reasons why the search for recognizable criteria of maturation in the lymphocyte has not been pressed. One must assume to begin with that among the mononuclear cells called "lymphocytes," some, if not all, arise as immature lymphocytes (lymphoblasts), and mature in a definite orderly way, to degenerate eventually without dedifferentiation. This assumption involves indorsement, at least in part, of the polyphyletic doctrine of cell evolution in relation to

the lymphocyte, as it is obviously necessary for a cell to have a definite life cycle without change to other cell types before any conception of an orderly maturation can be entertained. The justification for such an assumption in relation to the lymphocyte will be evident as the experimental data which are to follow are introduced.

Another difficulty of the problem lies in the physical character of the lymphocyte itself, in that there is no apparent elaboration of specific structures in the cytoplasm. The development of granules in the cytoplasm and of nuclear polymorphism in the granulocyte, of a rosette of vacuoles stainable with neutral red in the monocyte, of phagocytic vacuoles without pattern in the clasmatocyte, and of hemoglobin in the red blood cell have all served as objective criteria upon which to base identification and a study of maturative phases. The lack of comparable criteria has been an obstacle to the recognition of age states in the lymphocyte, accustomed as we are to look for specific products in specific cells. It is evident that criteria of a different character must be sought for.

A third difficulty is to be found probably in the physiology of the lymphocytogenic tissues. Because of their anatomical distribution, it is highly probable that many of the lymphocytes perform their function within the tissue in which they arise. We may expect functionally mature lymphocytes to be intermingled with cells of varying degrees of immaturity in lymph nodes. It is impossible on the basis of present knowledge to examine lymphatic tissue, as in the case of bone marrow, with the assurance that there will be a preponderance of immature types with which to compare those forms in the circulating blood.

Finally, the uncertainty which exists concerning the exact structure of lymphatic tissue contributes to the complexity of the problem. Although most authors believe that the germinal center of Flemming is the lymphocytogenic focus, others interpret such so called germinal centers as reaction areas which do not participate in the origin of the lymphocyte.

Possible Criteria of Age

The following characteristics of blood cells may conceivably be of use in estimating the age of lymphocytes.

1. Basophilia.—In 1898 Pappenheim (8) noted that the cytoplasm of young myelocytes is very basophilic, and since then the association of basophilia with youth of the granulocytes has been noted by many investigators. The same fact has been observed for red blood cells. The "reticulation" in young red blood cells is merely a precipitation of basophilic substance (1, 2, 9–11); and in the reticulated cell counts we have an example of the use of the presence of basophilia as an index of immaturity. A careful application of the criterion to lymphocytes has apparently not been made.

2. Mitochondria.—That mitochondria are present in large numbers during the maturation of young blood cells other than lymphocytes is generally accepted (12–14); and in an excellent review, Cowdry (15) states that senescence is associated with diminishing numbers of mitochondria in the cytoplasm of cells in general. It should be mentioned, however, that increased numbers of mitochondria are associated with the activity of secretory cells.

3. Size of the Cell.—Hematologists have most frequently used cell size as a criterion of the age of lymphocytes. But how uncertain this has proved is shown by the fact that some have regarded the large (16, 17) and some the small (7, 18–20) lymphocyte as the younger cell, while others hold that size is no criterion of age at all (21). It is significant that most of the investigators studying fixed, stained specimens adhere to the belief that the large cells are the older types, whereas those who follow the supravital technique hold the reverse.

4. Miscellaneous Features Characterizing the Lymphocyte.—Here may be listed motility, vacuoles, chromatin content of nucleus, proportion of nucleus to cytoplasm, shape of nucleus (indentations), and azur granulation. The possible significance of these characteristics will be brought out further on.

Evaluation of Criteria

On analogy with the other cells of the blood, an estimation of the mitochondria in the supravital technique and of the degree of basophilia in the fixed films would seem most likely to afford criteria of value in the estimation of the age of the lymphocyte.

To test out this possibility, it is necessary to analyze the lymphocytes with respect to these criteria (1) in bloods which may be expected to show a normal distribution of age types, and (2) in bloods obtained when a physiological or pathological hyperplasia of lymphoid tissue should lead to increased numbers of immature types in the circulation. Of the criteria, basophilia is the most easily and accurately determined.

1. Basophilia.—With the supravital technique (17, 22) basophilia is detected by a grayish yellow hue or cast to the cytoplasm. In very basophilic cells this color approaches the hemoglobin tint of mature red corpuscles. A difficulty exists, however, in estimating relative basophilia in all the lymphocytes in supravital preparations, because of the very small amount of cytoplasm in the small

cells. Only rarely can this criterion be applied except in intermediate and large lymphocytes, and since the small cell usually predominates, the factor of basophilia cannot be analyzed uniformly in the supravital preparations.

Fixed and stained films furnish the best means for studying basophilia. Nearly all of the Romanowsky dye combinations bring out the basophilia clearly; but a combination of Wright and Giemsa stains (23) most clearly indicates its finer degrees. The usual Wright's staining method is followed, without washing, by a floating of the cover slip, stained side down, in diluted Giemsa (15 drops Giemsa plus 10 cc. distilled water at pH 6.5–7.0) for about 4 minutes. The cover slips are then blotted and mounted. The cytoplasm of lymphocytes usually does not take the stain uniformly; there may be condensations and rarefactions or an appearance of granulation. This is probably due to a precipitation of the basophilic substance comparable to the "reticulation" of red cells. Care must be observed to select fields for counting in which the spread is very thin, with no cells overlapping. It is also of prime importance that the stain be working properly, as it is useless to attempt to grade cells on basophilia in a poorly stained specimen.

After accustoming the eye to the differing shades of blue which this stain imparts to the cytoplasm, one can divide the lymphocytes arbitrarily into three types: The very basophilic, the moderately basophilic, and the slightly or not at all basophilic. The first type (Figs. 1 and 2) reacts with a color such as that obtained when a mark is made on white paper with a blue crayon (hereafter designated as "Y" types); the second type (Figs. 3 and 4) is "sky blue" (designated "M" types); while the third (Figs. 5, 6, and 7) is tinged scarcely at all with blue or may even react with a faint pinkish tinge to the cytoplasm ("O" types). Because of the gradual transition between the shades of color, it may be difficult at times to classify a cell, but with a little experience one learns to divide them rather sharply into the three classes. The smallest fragment of cytoplasm in a small lymphocyte is sufficient to make the classification on the basis of basophilia.

As the first step toward evaluating basophilia as a criterion of age, an analysis of the lymphocytes in a series of normal adult human and rabbit bloods was made. A total of 100 to 200 cells of all types were counted in the fixed smears and a percentage computation was made for each of the three degrees of basophilia. Tables I and II give the data procured.

In Table I the results obtained in blood specimen R 612C are omitted from the averages, as it was found that this man, an X-ray worker, was subject to recurring periods of lymphopenia, a fact discovered as a direct result of the finding in this survey.

It will be seen that normal bloods, both human and rabbit, have relatively definite and stable percentage composition of basophilic

lymphocyte types. The average formula (Y-M-O) which expresses decreasing degrees of basophilia is for 13 human blood samples, 5-49-46. For 14 normal rabbits this formula is 4.7-48-47.3. As the tables show, the individual variation from these formulae was remarkably little. The number of specimens studied is insufficient to enable one to accept the formulae as representative of normal physiological

TABLE I

Basophilia and Comparative Size of Lymphocytes in Normal Human Blood

Specimen	Sex	Date	No. of lympho-cytes per c.mm.	Percentage of basophilic types Fixed technique			Percentage of size types Supravital technique		
				Y forms	M forms	O forms	Small	Int.	Large
R 1497*	F	May 21	1,768	9	48	43	96	4	0
R 1498	M	May 21	1,749	6	64	30	56	21	3
R 1499	M	May 21	869	5	55	40	88	12	0
R 1500	F	May 21	1,539	4	54	42	80	12	8
R 1501	M	May 21	2,240	6	47	47	82	15	3
R 1502	M	May 21	3,081	3	56	41	59	41	0
R 1503	M	May 21	4,238	4	65	31	98	2	0
R 169F	M	Apr. 25	3,360	7	34	59	72	28	0
R 612C	M	Apr. 25	896	17	58	15	100	0	0
R 612W	M	Apr. 25	1,716	3	36	61	76	24	0
R 612P	M	Apr. 25	1,470	6	69	25	70	30	0
R 612L	M	May 20	1,608	4	32	64	84	16	0
R 1493T	M	May 20	854	8	35	57	86	14	0
R 1493M	F	May 20	1,001	3	50	47	92	8	0
Average (R 612C omitted).....			1,960	5	49	46	82	17	1

* These are serial numbers of the work of the department covering a term of years.

standards, but they are adequate for the purposes of the present investigation.

The second step was to examine bloods which might be expected to show an increase in immature lymphocytes. Since in new-born animals immature myeloid and erythroid cells are regularly found in the circulating blood, it is logical to expect an unusually great proportion of immature lymphocytes. Examination was made of 12 samples of blood from 6 rabbits the ages of which varied from 12 hours to 19

days, and the lymphocytes were graded according to basophilia as before. Owing to the extreme leucopenia in the young rabbits, only 25 to 50 cells of all types were counted. Table III contains the data thus secured.

Considering for the moment only the column containing the basophilic types, it is noted that the average formula (Y-M-O) is 38.8-

TABLE II

Basophilia and Comparative Size of Lymphocytes in Blood of Normal Rabbits

Rabbit	Date	No. of lymphocytes per c.mm.	Percentage of basophilic types Fixed technique			Percentage of size types Supravital technique		
			Y	M	O	Small	Int.	Large
R 1129*	3/12–4/14	3,045	6.7	47	46.3	92.6	6	1.4
R 1130**	3/12–4/21	6,079	3.7	50.6	45.7	90.7	7.8	1.5
R 1134**	3/12–4/14	2,623	11.1	54.4	34.5	87.8	8.6	3.6
R 1131	5/20	2,968	3	53	44	72	28	0
R 1132	5/20	13,778	2	56	42	99	1	0
R 1133	5/20	2,275	7	45	48	94	3	3
R 1135	5/20	4,033	15	56	29	88	9	3
R 1136	5/20	3,549	2	49	49	89	11	0
R 1137	5/20	2,460	2	50	48	83	14	3
R 1138	5/20	1,396	7	53	40	90	10	0
R 1139	5/20	7,864	3	44	53	98	2	0
R 1167	5/20	7,260	0	40	60	92	3	5
R 1169	5/20	4,902	0	26	74	94	6	0
R 1170	5/20	2,004	3	45	52	96	4	0
Averages.....		4,590	4.7	48	47.3	90.5	8.1	1.4

* R 1129 represents average of 9 counts.

** R 1130 and R 1134 represent averages of 11 counts each.

53.3-7.9; that is, there is a marked shifting to the heavily basophilic types as compared with specimens of normal adult blood.

Blood specimens from 20 rabbits with tuberculosis were next examined for possible changes in the cellular formula (Table IV). These tuberculous rabbits have been divided into two groups on the basis of the monocyte-lymphocyte ratio in the peripheral blood; eleven in which the M/L index was less than 0.50, probably indicating a minimum of tuberculous activity (24, 25), and nine in which this index

was 0.50 or more, probably indicating progressive lesions. In the latter group it will be seen that there is a notable shift in the lymphocyte formula to the more basophilic types, 22 per cent, together with a relative lymphopenia, when compared with the group in which the M/L index was less than 0.50. In those cases in which the M/L ratio is highest (see R 1366, R 1309, and R 1103, in particular), the increase in number of basophilic lymphocytes is especially marked.

TABLE III

Percentage of Basophilic, Mitochondrial, and Size Types in the Blood of New-Born Rabbits

Rabbit	Age	Date	No. of lympho-cytes per c.mm.	Percentage of baso-philic types Fixed technique			Percentage of mitochondrial types Supravital technique			Percentage of size types Supravital technique		
				Y	M	O	Y	M	O	Small	Int.	Large
R 1377	4 days	3/8	864	15	78	7	38	38	24	70	30	0
R 1377	10 "	3/15	1,100	50	46	4	0*	100	0	88	12	0
R 1377	19 "	3/24	2,400	43	53	4	42	53	5	84	14	4
R 1378	4 "	3/8	810	47	47	6	0*	70	30	90	10	0
R 1378	10 "	3/15	840	59	36	5	24	62	14	95	5	0
R 1378	19 "	3/24	605	32	63	5	29	61	10	90	5	5
R 1379	4 "	3/8	594	30	65	5	0	57	43	34	66	0
R 1379	10 "	3/15	592	35	53	12	25	69	6	94	6	0
R 1379	19 "	3/24	1,620	46	32	22	15	55	30	96	4	0
R 1418	12 hrs.	3/31	192	25	50	25	25	50	25	100	0	0
R 1419	12 "	3/31	320	44	56	0	42	58	0	88	12	0
R 1420	12 "	3/31	260	40	60	0	0*	100	0	100	0	0
Averages.....			850	38.8	53.3	7.9	20.0	64.5	15.5	85.7	13.6	0.7

* Note that the difficulty in accurately estimating large numbers of mitochondria sometimes results in many or all the "Y" types being classified with the "M" types. This remark also applies to Table VI.

In general, this relationship between basophilia and the M/L index persists throughout the table.

Since it is generally believed that the lymphocyte is an important element in resistance to tuberculosis, it is logical to assume that in cases progressing unfavorably, cases that is to say with a high M/L index, the lymphopenia reflects a demand for lymphocytes which the lymphatic tissues cannot adequately meet, and under such circumstances

the appearance in the blood stream of less mature types may be expected and is easily understood. In formulating this hypothesis, I am

TABLE IV

Percentage of Basophilic and Size Types of Lymphocytes in the Blood of Tuberculous Rabbits

Rabbit	M/L index	Date	No. of lympho-cytes per c.mm.	Percentage of basophilic types Fixed technique			Percentage of size types Supravital technique		
				Y	M	O	Small	Int.	Large
R 1366	1.25	4/29	1,680	31	62	7	88	12	0
R 1309	1.21	4/21	2,590	53	41	6	93	7	0
R 1103	1.00	4/21	2,268	24	48	28	90	5	5
R 1152	0.93	5/19	2,380	13	60	27	84	16	0
R 1248	0.80	5/28	3,680	15	60	25	85	12	3
R 1262	0.60	4/28	4,865	9	52	39	94	3	3
R 1260	0.56	5/28	3,888	10	52	38	80	17	3
R 1381	0.56	4/28	1,927	18	53	29	90	5	5
R 1367	0.50	4/29	3,710	25	65	10	97	3	0
R 1383	0.43	4/28	3,220	11	57	32	83	17	0
R 1259	0.35	5/28	2,793	14	50	36	70	27	3
R 1112	0.33	4/30	3,663	13	38	49	76	24	0
R 1103	0.31	5/28	1,856	10	50	40	87	13	0
R 1184	0.22	5/28	2,808	13	44	43	91	7	2
R 1255	0.18	5/28	8,673	1	36	63	100	0	0
R 1389	0.18	4/28	3,753	11	63	26	93	7	0
R 1179	0.16	5/28	4,434	15	50	35	90	10	0
R 1269	0.15	5/28	3,054	5	52	43	93	7	0
R 1382	0.12*	4/1	5,964	26	60	14	60	30	10
R 1176	0.10	5/28	7,735	4	47	49	88	12	0
Average of all rabbits	0.497		3,747	16.0	52.0	31.9	86.6	11.7	1.7
Average of group with M/L index of 1.25–0.50 (9 animals).....			2,998	22.0	54.7	23.2	89.0	8.8	2.1
Average of group with M/L index of 0.43–0.10 (11 animals)..			4,359	11.1	49.7	39.0	84.6	14.0	1.3

* Rabbit R 1382 died 2 weeks after this blood count was taken, indicating that this animal probably had advanced tuberculosis.

merely applying to the lymphatic tissues the same reasoning which has been applied to the myeloid tissues and found true in the case of pyogenic infections.

Further evidence to show that increasing basophilia is a criterion of increasing immaturity was obtained by the examination of blood from animals in which lymphoid hyperplasia has been artificially induced. Figs. 10–15 show the degree of hyperplasia induced in the lymphatic tissues of rabbits receiving parenteral injections of foreign protein (26). They show a multiplication of lymph follicles both in lymph nodes and in the spleen, together with an increase in basophilic forms and in mitotic figures in these tissues. The blood counts of these same animals are given in Table V.

The left half of Table V shows the extent of increase in the numbers of lymphocytes per cubic millimeter during the period of the injections. The basophilic formula at various times during the period of the injections is shown in the right hand section of the table. Expressed as an average of 25 examinations of the blood from 5 animals, this formula is 27.1-46.9-26, which again shows a marked shift to the more basophilic types. If the hypothesis is correct that the peripheral blood reflects the state of activity of the tissue from which its cells are derived, then it must be concluded that basophilia is a measure of immaturity of the lymphocyte.

2. *Mitochondria.*—If it be accepted on the basis of the above evidence that basophilia is directly related to youth in the lymphocyte, then it becomes possible to see if any other property, such as a high mitochondrial content, is linked with youth.

Blood specimens in normal adult, new-born, and tuberculous rabbits were examined for the purpose of obtaining direct comparison between these two factors. The intensity of basophilia was determined in Wright-Giemsa-stained specimens and mitochondrial content in supravital preparations. To preclude obtaining specimens of blood for comparison that might be dissimilar with respect to the lymphocytes, both preparations were made from portions of the same drop of blood by drawing it into the stem of a white cell diluting pipette and expelling small portions very rapidly, first for the supravital and then for the fixed preparations.

Mitochondrial content was determined by count whenever possible,

at other times by rough estimation; and the classification was made as follows: Class Y represents cells with many mitochondria, that is, more than 20; Class M includes cells with from about 5 to 20 mitochondria,

TABLE V

Percentages of Basophilic Types of Lymphocytes in Rabbits with a Hyperplasia of the Lymphatic Tissues Induced by Protein Injections

Rabbit	Average Nos. of lymphocytes per c.mm. of blood during control and injection periods with date of autopsy		Date of count	Per cent basophilic types during injection period Fixed technique		
				Y	M	O
R 1157	Control period	2,477	2/21/30	27	51	22
	Injection period	3,911	2/26/30	59	30	11
	Date of autopsy	3/27/30	3/14/30	25	50	25
			3/19/30	27	59	14
			2/24/30	23	42	35
			3/26/30	24	39	37
R 1158	Control period	3,507	2/10/30	32	45	23
	Injection period	5,151	2/11/30	30	52	18
	Date of autopsy	2/21/30	2/19/30	33	38	29
			2/21/30	38	43	19
R 1160	Control period	3,179	2/26/30	37	43	20
	Injection period	5,599	3/ 7/30	20	51	29
	Date of autopsy	3/20/30	3/10/30	10	41	49
			3/14/30	23	40	37
			3/19/30	32	43	25
R 1163	Control period	3,208	2/14/30	20	57	23
	Injection period	6,500	3/ 7/30	16	60	24
	Date of autopsy	4/ 4/30	3/10/30	20	47	33
			3/19/30	18	54	28
			3/24/30	15	52	33
			3/26/30	31	47	22
			4/ 2/30	18	54	28
			4/ 4/30	13	46	41
R 1185	Control period	2,504				
	Injection period	5,983	4/25/30	62	28	10
	Date of autopsy	6/20/30	4/28/30	25	59	16
Averages....	Control period	2,975		27.1	46.9	26
	Injection period	5,429				

and Class O cells with from 0 to 5. The corresponding groups of lymphocytes arranged according to basophilia of the cytoplasm as determined in the fixed films were classified as before; that is, Class Y, deeply basophilic, Class M, moderately basophilic, and Class O, slightly or not at all basophilic. Tables III and VI present the data so obtained.

It is impossible to obtain accurate estimations of mitochondrial content, partly because of the physical difficulties involved in counting these bodies and partly because of the fact that some of the mitochondria occasionally fail to take the dye, while some fading of the stain often occurs. Tables III and VI are therefore presented to show trends, not exact quantitative relationships. As such, the averages in these tables demonstrate clearly that when the trend of the lymphocytes is towards increased cytoplasmic basophilia there is also a corresponding shift towards increased mitochondrial content. This same fact is demonstrated in Table VIII, Columns 6 and 7, in which basophilia and mitochondrial content of each of 75 large lymphocytes are directly compared by supravital examination.

3. Size of the Cell.—With the demonstration that increasing mitochondrial and basophilic content of the cytoplasm of lymphocytes are found together and that they are criteria of increasing immaturity, it becomes important to test the relation that cell size bears to these indices. The facts to be determined are: (1) The relation, if any, between increasing immaturity and the increase or decrease in numbers of the large cells, and (2) the determination of the status of the large lymphocyte with respect to its position in the maturative cycle. As a prerequisite for this study it was necessary first, to consider the measurements obtained as such, and second, to determine if the procedure of pulling and fixing cover slips distorts the size and appearance of the cell.

Among many hundreds of lymphocytes measured, the smallest encountered was 5.2 micra in diameter, and the largest 20.0 micra. The usual allocation of size to type has been given as 6μ–9μ for the "small" lymphocyte, 9μ–12μ for the "intermediate," and $12\mu+$ for the "large" lymphocyte (16, 20, 21, 27). It was found, however, that a great number of cells, when studied by the supravital method, measured slightly over 9μ, with very few between this size and 10.5μ.

For this reason the following standards of size were adopted in the present study: up to 10μ the small lymphocyte, 10μ–12μ the intermediate, and 12μ+ the large lymphocyte.

TABLE VI

Comparison by Percentage of Mitochondrial and Basophilic Types of Lymphocytes in Three Normal and Five Tuberculous Rabbits

Rabbit	Date	Pathology	Percentage of basophilic types Fixed technique			Percentage of mitochondrial types Supravital technique		
			Y	M	O	Y	M	O
R 1129	3/12	None	7	50	43	3	55	42
R 1129	3/14	"	4	46	50	3	51	46
R 1129	3/17	"	10	44	46	6	73	21
R 1129	3/19	"	5	47	48	5	47	48
R 1129	3/24	"	7	57	36	7	57	36
R 1129	3/28	"	6	45	49	8	52	40
R 1129	3/31	"	10	37	53	4	61	35
R 1129	4/2	"	5	52	43	5	52	43
R 1130	3/12	"	3	60	37	20	60	20
R 1130	3/14	"	8	61	31	0	50	50
R 1130	3/17	"	2	48	50	6	60	34
R 1130	3/19	"	2	55	43	3	58	39
R 1130	3/24	"	5	56	39	3	65	32
R 1130	4/2	"	5	49	46	8	55	37
R 1134	3/12	"	11	46	43	18	43	39
R 1134	3/14	"	12	62	26	0	74	26
R 1134	3/17	"	20	47	33	9	69	22
R 1134	3/19	"	17	60	23	17	61	22
R 1134	3/24	"	16	43	41	8	60	32
R 1134	4/2	"	12	50	38	6	63	31
R 1185	4/25	Tbc.	62	28	10	24	62	14
R 1176	4/2	"	30	60	10	0	80	20
R 1366	4/29	"	31	62	7	18	75	7
R 1367	4/29	"	25	65	10	30	61	9
R 1382	4/1	"	26	60	14	11	60	29
Averages for R 1129.....			6.7	47.2	46.1	5.1	56.0	38.9
Averages for R 1130.....			4.1	54.8	41.1	6.6	58.0	35.4
Averages for R 1134.....			14.6	51.3	34.1	9.6	61.6	28.8
Averages for tbc. rabbits.....			34.8	55.0	10.2	16.6	67.6	15.8

Within limits, there is little or no relation between the size of the lymphocyte as obtained in the fixed smear and the real size of that cell in the circulating blood. Evidence in support of this statement is offered in Table VII. This represents a comparison of the measured gross diameters of the lymphocytes encountered in five separate differential counts of five different normal human bloods. Portions

TABLE VII

Number of Lymphocytes of Various Sizes Found in Counting 100 White Blood Cells in Supravital and Fixed Preparations of Blood from Five Normal Human Adults

Blood specimen	Technique	Gross cell diameters in micra														
		5–6	6–7	7–8	8–9	9–10	10–11	11–12	12–13	13–14	14–15	15–16	16–17	17–18	18–19	19–20	20+
R 1497	Supravital	—	6	13	3	4	1	—	—	—	—	—	—	—	—	—	—
Female–age 58	Fixed	—	—	—	1	8	1	4	3	2	6	2	—	—	—	—	—
R 1498	Supravital	—	—	6	8	11	6	1	1	—	—	—	—	—	—	—	—
Male–age 28	Fixed	—	—	—	4	5	4	10	7	0	2	1	—	—	—	—	—
R 1499	Supravital	1	2	2	3	6	0	2	—	—	—	—	—	—	—	—	—
Male–age 31	Fixed	—	—	—	—	1	2	4	6	1	2	1	—	—	—	—	—
R 1500	Supravital	—	3	9	1	8	1	3	1	0	1	—	—	—	—	—	—
Female–age 27	Fixed	—	—	—	1	3	4	10	6	0	2	1	—	—	—	—	—
R 1501	Supravital	2	4	8	6	6	0	4	0	0	1	—	—	—	—	—	—
Male–age 33	Fixed	—	—	—	1	5	2	6	6	2	6	2	0	0	0	2	—
Totals	Supravital	3	15	38	21	35	8	10	2	0	2	—	—	—	—	—	—
	Fixed	—	—	—	7	22	13	34	26	5	18	7	0	0	0	2	—
Per cent	Supravital	83					13		4								
	Fixed	20					35		45								

from the same drop of blood were used in making both supravital and fixed spreads.

It is obvious that there is a great preponderance of large forms in the fixed films that does not occur in supravital preparations. Thus, only 4 per cent of the lymphocytes in the living state measured over 12 micra, whereas in the fixed films the corresponding percentage was 45.

This means, that in viewing a large lymphocyte in the fixed preparation, one cannot tell the size of that cell when in the circulating blood. The explanation is suggested that some cells, being more fragile than others, are flattened out by the technique usual in pulling cover slips with consequent distortion of size. Others, being more elastic and coherent, resist more effectually this mechanical factor. In cases of decrease in cell size, it can be supposed that contact with fixative has resulted in shrinkage of the cells.

Having determined that the size of cells in fixed specimens is not comparable with that of living ones, it became necessary to compare the percentages of the three cell sizes, as determined by supravital examination, with the percentages of the basophilic types obtained in fixed films, if one was to establish any relation between size and state of maturity. Such comparisons in bloods from normal human adults (Table I), normal adult rabbits (Table II), new-born rabbits (Table III), and tuberculous rabbits (Table IV) show that an increase in immature forms may occur without any notable shift in size. However, an analysis of these tables indicates that in some instances an increase in young forms was associated also with an increase in the larger types of cells (compare average of size types in Tables II, III, and IV and individual counts of R 1377 and R 1378 on March 24 of Table III, and R 1382 on Table IV).

A study of large lymphocytes was now undertaken to determine if they are predominately young or old. To determine this point, 75 large lymphocytes were measured in supravital specimens and observations were also made on their degree of basophilia and mitochondrial content and on certain other features of the living cell. The results obtained are shown in Table VIII, Columns 2, 6, and 7.

Analysis of Table VIII indicates that most but not all of the large lymphocytes have a marked basophilia and contain large numbers of mitochondria. Those that are deeply basophilic and with nearly maximum numbers of mitochondria (+++ and ++++) make up 70 per cent of the large lymphocytes examined, whereas the cells deficient in these respects (0 and 1+) constitute only 15 per cent of those surveyed. As an example of the latter group, a very large cell (No. 27), measuring 16.1μ, had a cytoplasm entirely deficient in signs of basophilia and mitochondria. Since these two characteristics,

TABLE VIII

Supravital Characteristics of Large Lymphocytes in the Blood of Rabbits

Cell No.	Gross diameter	Approximate ratio of diameter of nucleus to cell	Chromatin content of nuclei	Approximate depth of indentation of nucleus	Basophilia of cytoplasm	Mitochondria	Neutral red vacuoles	Nucleoli	Non-staining refractive vacuoles
				μ					
1	14.9	0.90	++	F	++++	++++	+	2	0
2	13.3	0.60	++	F	++++	++++	++	0	0
3	15.0	0.70	++++	1.6	+++	+++	+	0	0
4	15.0	0.55	++++	Round	++++	++++	++	0	0
5	14.5	0.60	+++	2.6	++++	+++	+	0	0
6	16.6	0.75	++	1.8	++++	++++	++	0	0
7	15.0	0.70	++	1.2	+++	++	+	0	0
8	17.5	0.60	++++	0.5	++++	++++	++	0	0
9	14.5	0.70	++	0.5	++++	+++	++	0	0
10	15.0	0.80	++++	1.0	+	++	+++	0	0
11	13.2	0.55	++	1.6	+++	++	++	0	0
12	15.7	0.60	++	0	++++	+++	+	0	0
13	12.8	0.60	+	1.6	++++	++	+++	0	0
14	15.0	0.88	++++	Round	+++	+++	++	0	0
15	16.0	0.70	+	Round	++++	+++	+++	0	0
16	15.0	0.70	++	F	++++	++++	+	1	0
17	14.5	0.65	++++	2.2	++++	++++	++	0	0
18	13.0	0.75	+	1.5	+++	+++	++	0	0
19	13.0	0.75	++++	0	+++	+++	++	0	0
20	13.0	0.75	++++	F	+++	+++	++	0	0
21	16.0	0.60	++++	Oval	++++	++++	++	0	0
22	13.0	0.60	+	F	++	++	+	0	0
23	15.0	0.75	++	5.0	+++	++	+	0	0
24	14.0	0.70	++	4.0	++++	+++	++	0	0
25	19.5	0.90	++	4.5	++++	++++	+++	0	0
26	14.4	0.75	+	Round	++	++	+++	0	0
27	16.1	0.30	+++	Round	0	0	0	0	1
28	15.0	0.70	++	F	++	++	+	0	0
29	14.5	0.75	++	5.0	+++	+++	++	0	0
30	16.0	0.70	++	0.5	++++	++++	+++	0	0
31	14.5	0.70	++	2.0	+++	+++	+	0	0
32	14.4	0.45	+	Round	0	0	++	0	++++
33	15.0	0.50	+	F	+	+	++++	0	+
34	20.0	0.75	++	Oval	++++	++++	+++	0	0
35	16.5	0.95	++	Round	++++	++++	+++	1	0
36	13.0	0.85	++	Round	++++	+++	0	0	0
37	16.0	0.90	++	F	+++	+++	+++	0	0
38	14.5	0.50	+	Round	0	0	0	0	++++

F = flattened.

TABLE VIII—*Concluded*

Cell No.	Gross diameter	Approximate ratio of diameter of nucleus to cell	Chromatin content of nuclei	Approximate depth of indentation of nucleus	Basophilia of cytoplasm	Mitochrondria	Neutral red vacuoles	Nucleoli	Non-staining refractive vacuoles
				μ					
39	16.0	0.75	+	Oval	++	++	++++	0	0
40	16.1	0.55	+	2.5	++	++	++++	0	0
41	19.0	0.80	++++	F	++++	++++	+++	0	0
42	14.4	0.90	++	1.0	++++	++++	++	0	0
43	15.0	0.60	++	Oval	+++	+++	++	0	0
44	14.5	0.70	++++	1.0	+++	+++	++	0	0
45	13.5	0.85	+++	Oval	++++	++++	+	0	0
46	15.0	0.75	+	F	+++	++	++	0	0
47	13.0	—	—	—	++++	++++	0	0	0
48	13.5	0.70	++	5.0	++	++	++++	0	0
49	16.0	0.55	+++	4.0	++++	++++	0	0	0
50	16.0	0.60	+	4.0	++	++	+	0	0
51	13.0	0.60	++	Oval	++++	++++	+	0	0
52	13.0	0.70	++	F	++	++	++	0	0
53	13.0	0.60	++	F	+	+	++++	0	++++
54	13.0	0.70	++	5.0	++	++	++++	0	0
55	15.0	0.65	++	2.0	++	++	++++	0	0
56	13.0	0.70	++	Round	++++	++++	+++	0	0
57	12.8	0.70	+	F	0	0	++++	0	0
58	15.0	0.70	++	F	++++	++++	+++	0	0
59	12.8	0.80	++++	Round	++++	++++	0	1	0
60	14.5	0.55	+++	Round	0	0	++	0	+
61	12.8	0.70	++	Oval	++++	++++	0	0	0
62	17.5	0.65	+++	Round	+++	+++	++	0	0
63	12.0	0.70	++	Oval	++	++	++++	0	0
64	16.0	0.75	++	Oval	++++	++++	+	0	0
65	14.5	0.70	++	F	+++	+++	++++	0	0
66	14.5	0.75	++	F	+++	+++	++++	0	0
67	14.5	0.60	++	3.0	+++	+++	++++	0	0
68	14.5	—	—	—	++++	++++	+	0	0
69	13.0	0.70	+++	Oval	+	+	++++	0	++
70	11.2	0.60	+++	Round	0	0	+	0	++++
71	14.5	0.60	+++	F	++	++	++	0	0
72	13.0	0.65	+	Oval	+	0	0	0	++
73	14.4	0.70	++	Oval	+++	+++	++	0	0
74	14.5	0.90	++++	Round	++++	++++	++	1	0
75	16.0	0.70	++++	Round	+++	+++	++	0	0

Cell 69 changed to Cell 70 in 15 minutes under observation.
Cell 72 was watched for 1½ hours and no change in morphology occurred.

when present, have been shown to be associated with immaturity, it can be concluded that, although the majority of large lymphocytes of the supravital films are young, cells may be large at any age. The same holds true for the erythrocyte and leucocyte.

4. Miscellaneous Features Found in Lymphocytes.—Amongst the features detectable in the lymphocytes of supravital preparations, there remain to be evaluated the shape of nucleus (indentations), the relative size of nucleus to gross cell size, chromatin content of the nucleus, non-staining refractive vacuoles, and content of neutral red vacuoles. For the association of these features with mitochondrial content, basophilia, and cell size, reference is again made to Table VIII. The number of neutral red bodies, mitochondria, and refractive vacuoles were counted or estimated on a basis of 0 to 5 = +; 5 to 15 = ++; 15 to 30 = +++; over 30 = ++++. The amount of indentation of nucleus was measured or estimated; basophilia and amount of chromatin in the nucleus were estimated as +, ++, +++, and ++++.

Analysis of Table VIII with respect to these features indicates: (*a*) That the shape of the nucleus of the lymphocyte (presence of indentation and its degree) does not bear any relationship to the number of mitochondria or the degree of basophilia, and hence is unrelated to age in direct contradiction to Arneth's hypothesis (7), whereas the chromatin content of the nucleus does appear to be greatest in those cells deficient in the cytoplasmic criteria of youth. (*b*) That in general, in the large lymphocytes, the greater the amount of cell occupied by the nucleus, the more basophilic the cytoplasm. (*c*) That nucleoli are visible only in the nuclei of cells with deeply basophilic cytoplasm and sparse chromatin. It is probable that only large nucleoli are visible by this method of examination, as very small nucleoli are likely to be obscured from view by nuclear substance. (*d*) That non-staining, refractive vacuoles are found only in cells devoid of both mitochondria and basophilic substance; whereas the presence and number of neutral red vacuoles do not seem to bear any relationship to either of these features.

Azur granulation is seen in fixed preparations, but not in the supravital. The observations made in the present study have indicated that these granules are associated most often with the lesser basophilic

types. However, one cannot be too certain that they are entirely limited to this older cell type, because the dark staining of the cytoplasm in the young basophilic cells may make it impossible to detect them when present. The fact that no cytoplasmic structure of the living lymphocyte can be identified with the azur granule of the dead cell suggests that the latter is a precipitation brought about by fixation, possibly evidencing some functional activity of the mature cell.

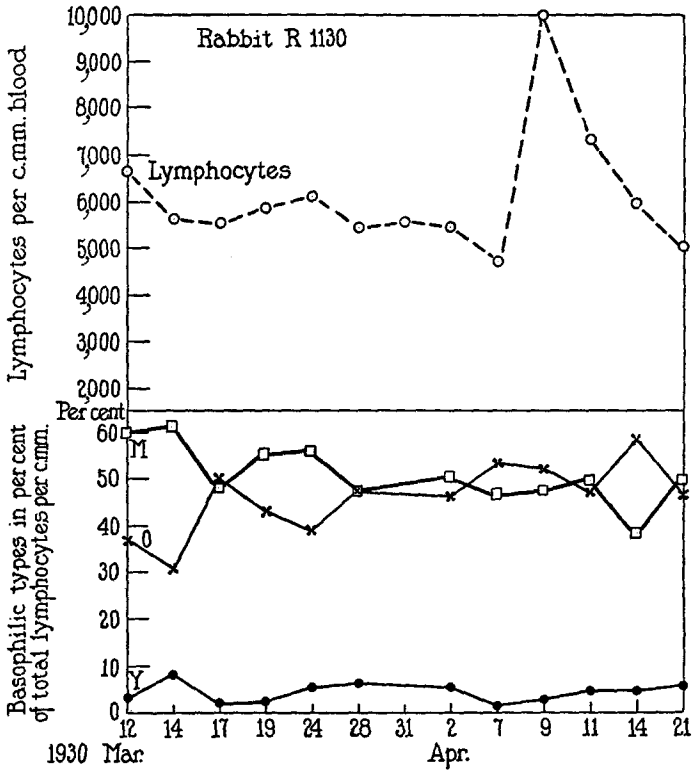

CHART 1. There are four curves on this chart to be contrasted. The uppermost shows the fluctuations in total number of lymphocytes; the curve labelled "O" designates the percentage of old cells (least basophilic); "M" refers to the mature (moderately basophilic) types; while the curve "Y" at the bottom of the chart indicates the percentage of young or heavily basophilic types.

Relation of Lymphocytosis to Cellular Immaturity

Arneth's dictum that the total number of granulocytes in the blood is not necessarily a criterion of the activity of the bone marrow has

been amply confirmed. It is of interest to analyze the number of lymphocytes circulating in the peripheral blood as an index to activity of the lymphoid tissue. Since it is now evident that the greater the basophilia the greater the immaturity of lymphocytes, one is enabled to make the analysis mentioned by comparing directly the percentage of the basophilic types with the total numbers of circulating cells. For this purpose, observations were made three times per week for 1 month on the lymphocytes in the blood of three stock rabbits. Chart 1 represents the findings in one of these animals. It is typical of all three.

Chart 1 shows clearly that the total number of lymphocytes in the peripheral blood is not necessarily a criterion of a percentage increase therein of young cells. Note especially that the high peak in total lymphocytes on April 9th showed no corresponding fluctuation in the percentage of basophilic types.

DISCUSSION

The present study shows that in the maturing lymphocyte changes take place in number of mitochondria and degree of basophilia, which have the same general character as those occurring during the life of the other cells of the blood.

Although basophilia is manifestly a criterion of youth, the absence of it in a given cell does not necessarily mean that the cell is mature. No one criterion enables one to pronounce upon this point. In other cell strains some of the recognized characteristics of youth may be absent in elements of undoubted immaturity. For example, occasionally there are seen early myelocytes that are motile (about 60 per cent in one case of myelocytic leucemia recently observed); mature neutrophilic leucocytes with very few specific granules; myelocytes without appreciable numbers of mitochondria or with little basophilic substance. From this experience with cell types other than the lymphocyte, it would seem that in the case of the lymphocyte as in that of other cell types, all of the criteria of immaturity must be taken into account in an age classification. Basophilia is the most constant and reliable criterion.

It now seems certain that each strain of blood cells can be traced back to a simpler form characterized by a more basophilic cytoplasm.

The widespread distribution throughout the body of basophilic mononuclear cells gave rise to Pappenheim's concept of a "lymphoidocyte" or indifferent polyvalent primitive cell. The monophyletic theory of cell origin has been based upon this conception. Although the decreasing basophilia which characterizes the cytoplasm of the lymphocyte from youth to senility is wholly analogous to that seen in the other types of developing white blood cells, nevertheless the demonstration of an early basophilic form in this series, need not necessarily be interpreted as support for the monophyletic hypothesis.

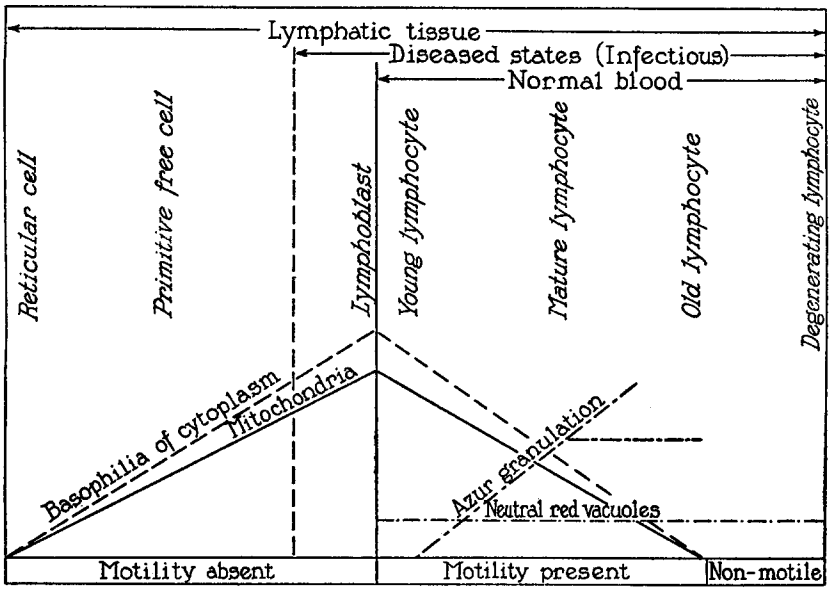

CHART 2. Diagram of the maturation of lymphocytes

The dualists have always held that each cell strain has its own "cytoblast," which matures only in the lineage peculiar to that specific cell type, while the monophyletic school have given the "lymphocyte" at all times multipotential powers of differentiation. The recognition of lessening basophilia of the lymphocyte until in the degenerating form the cytoplasm shows no basophilia whatever, establishes the lymphocyte as an independent definitive cell strain existing in parallel with the other blood cells, not preliminary to them. It will be recalled

that such an assumption was necessary to the undertaking of the present work; and it has been justified. A graphic representation of the changes taking place during the life of the lymphocyte is now possible. Chart 2 is such a representation, patterned after the schema of Sabin (13) for the red cells and granulocytes.

The failure to recognize basophilia as an expression of youth of the lymphocyte has often resulted in a designation of young forms as plasma cells. All possible gradations in basophilia have been encountered within the lymphocytic series as is indicated by Chart 2.

Unna's original concept (28) of the plasma cell included numerous basophilic forms. Von Marschalkó (29), however, sharply limited this term to a specific group of cells showing a relatively small nucleus excentrically placed, containing chromatin particles usually arranged radially (*Radkern*), with a central clear area in the protoplasm opposite the nucleus, and with very basophilic cytoplasm. The irritation forms of Türk (18) are usually considered as plasma cells (Naegeli (21)), but obviously if one accepts the definition of von Marschalkó these cells cannot be plasma cells.

Arneth's expression "shift to the left" (4) had its inception in the observation that under pathological conditions there occurs an increase in the proportion of those granulocytes showing fewer lobes to the nucleus, *i. e.*, younger cells. In a larger sense, "shift to the left" has come to express a shift toward the younger stages in the maturation cycle of some other blood elements, and applied in this broader sense, it is highly useful. When there occurs an increase in the proportion of older forms, the term "shift to the right" is applicable. It is evident from the present work that the terms can be used with respect to the lymphocyte.

SUMMARY

The study of blood from rabbits with normal and with hyperactive lymphatic tissue reveals, in the latter, a greater percentage of lymphocytes with heavily basophilic cytoplasm and numerous mitochondria. This indicates that cytoplasmic basophilia and mitochondrial content can serve as criteria of the degree of maturity of the lymphocyte, these characters having the same significance in this relation as obtains with other blood cells. Basophilia is the more evident and reliable indicator of youth of the cells. The classification of lymphocytes into

three groups, according to degree of basophilia, has yielded figures which show the proportions of the three to be relatively stable in blood from normal adult human beings and rabbits.

Size is not strictly a function of age in lymphocytes. Moreover, there is no correspondence in the size of lymphocytes in supravital films and in fixed specimens obtained by the "cover glass" method. There is a change of size during fixation. Although lymphocytes of intermediate and large size may be of any age, in supravital preparations the majority are young cells, whereas in fixed films the reverse obtains. The small lymphocyte may be of any age in specimens examined by either technique.

The total number of lymphocytes circulating at any given time is not necessarily an index to lymphoid activity.

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EXPLANATION OF PLATES

PLATE 25

FIG. 1. A large, very young lymphocyte from the peripheral blood of Rabbit R 1185, Apr. 25, 1930. Wright-Giemsa stain. Observe the granular character and extreme degree of basophilia of the cytoplasm. This is the "Y" type of cell mentioned in the text. Diameter of cell was 16.0 μ.

FIG. 2. A small lymphocyte showing the same characters. From Rabbit R 1381, Apr. 28, 1930. Diameter of cell was 8.5 μ.

FIG. 3. An intermediate lymphocyte from the peripheral blood of Rabbit R 1185, Apr. 25, 1930. Wright-Giemsa stain. Note the color of the cytoplasm. This is the "M" type of cell described in the text. Diameter of cell was 11.5 μ.

FIG. 4. A large lymphocyte showing the same features. From peripheral blood of Rabbit R 1185, Apr. 25, 1930. Diameter of cell 16.0 μ.

FIG. 5. A large lymphocyte from the peripheral blood of Rabbit R 1152, May 19, 1930. This is an old cell and is designated as the "O" type in the text. Note the lack of basophilia of the cytoplasm. One azur granule is to be seen near the top of the cell. Wright-Giemsa stain. Diameter of cell was 16.0 μ.

FIG. 6. An intermediate lymphocyte showing the same features. From peripheral blood of Rabbit R 1152, May 19, 1930. Diameter of cell was 11.8 μ.

FIG. 7. A small lymphocyte showing the same features. From the peripheral blood of Rabbit R 1176, May 28, 1930. Diameter of cell was 8.0 μ.

FIG. 8. A red blood cell from the peripheral blood of a rabbit stained by the Wright-Giemsa technique,—for purposes of comparison.

PLATE 26

FIG. 9. Section of inguinal lymph gland of Rabbit R 1160. Removed at biopsy before protein injections. Normal gland throughout. Hematoxylin and eosin. $\times$27.

FIG. 10. Section of inguinal gland of Rabbit R 1160 after 45 daily intravenous injections of chick embryo extract, showing an increase in size and number of secondary nodules and an increase in internodular lymphatic tissue. Compare size and histology of this gland with that of Fig. 9 and note cellular response in the former. Hematoxylin and eosin. ×27.

FIG. 11. Section of inguinal gland of Rabbit R 1158 after daily subcutaneous injections of chick embryo extract. The very hyperplastic gland consisted largely of diffusely distributed lymphatic tissue. This was rich in lymphoblasts with heavily basophilic cytoplasm. Hematoxylin and eosin. ×27.

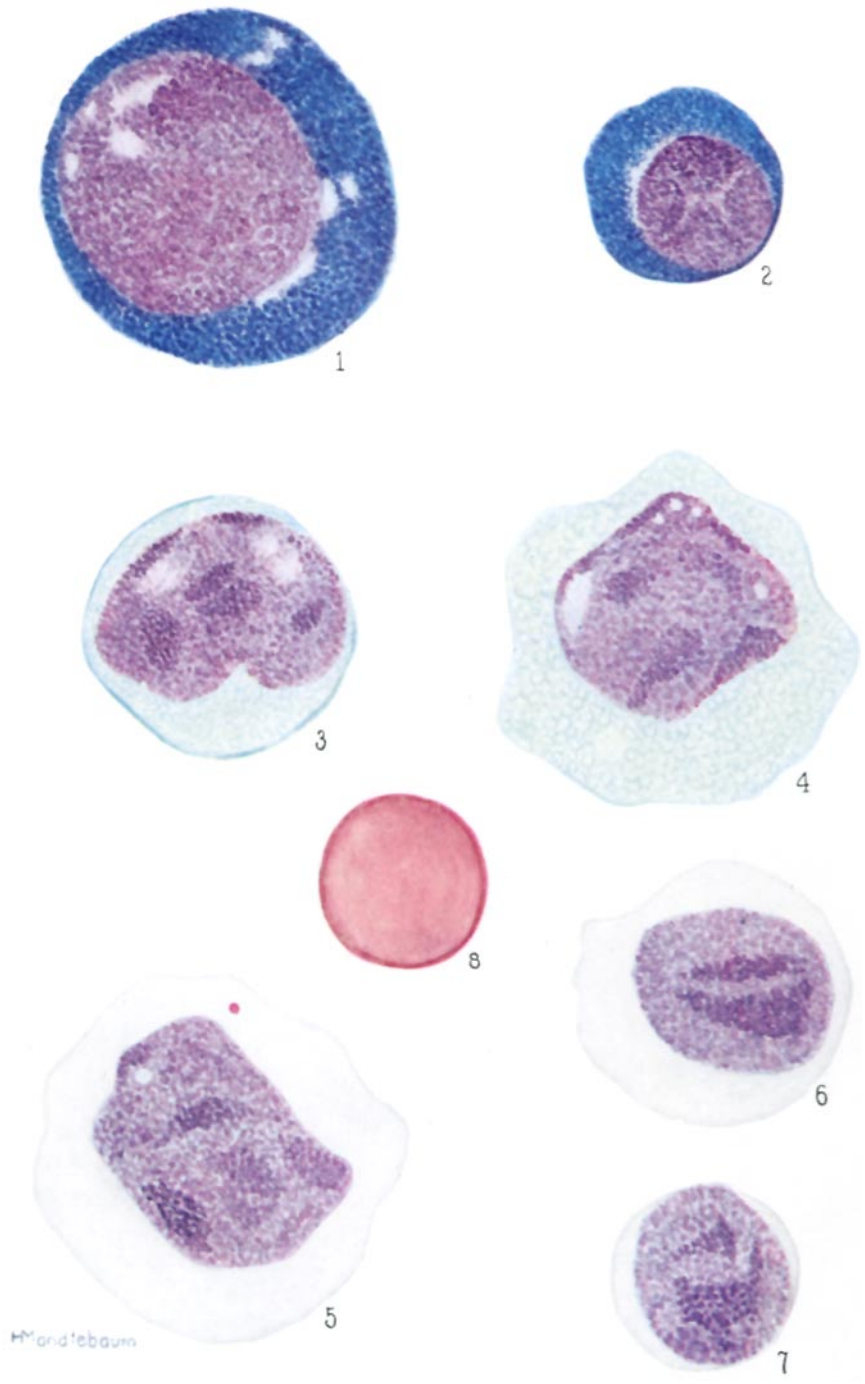
PLATE 27

FIG. 12. Section of spleen of control Rabbit R 1186 after 37 daily intravenous injections of normal salt solution. No evidence of hyperplasia. Hematoxylin and eosin. ×27.

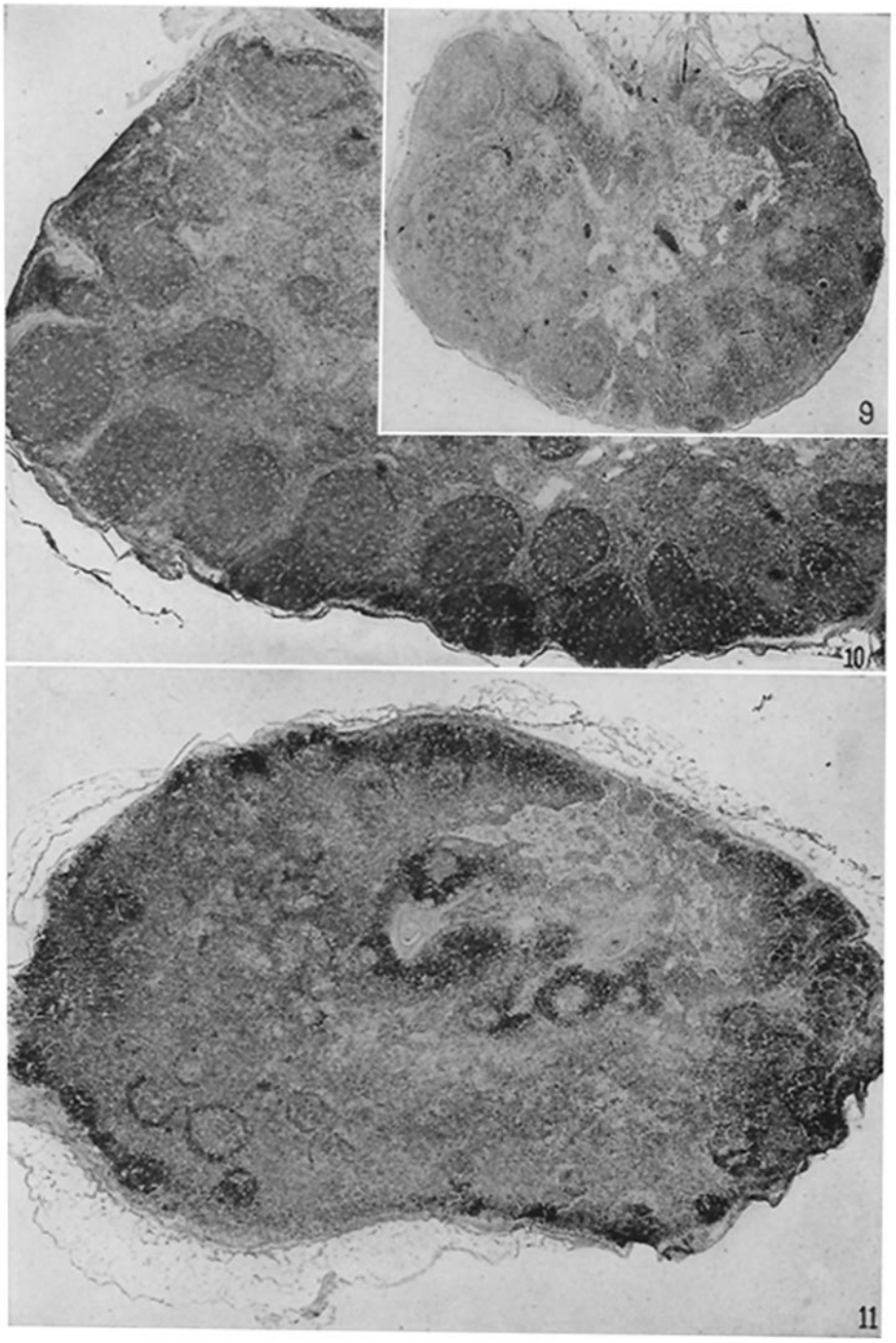
FIG. 13. Section of spleen of Rabbit R 1157 after 19 daily intravenous injections of chick embryo extract. Note the increase in size and number of Malpighian bodies as compared with control spleen of Fig. 12. Hematoxylin and eosin. ×27.

FIG. 14. Section of spleen of Rabbit R 1163 through germinal center. This animal received 25 daily intravenous injections of a solution of egg albumen. Note increase in number of mitotic figures and large cells with basophilic cytoplasm, typical of the spleens from all rabbits receiving these injections. Hematoxylin and eosin. Oil immersion.

FIG. 15. Section of spleen of Rabbit R 1160 through germinal center. This animal received 45 daily intravenous injections of chick embryo extract. Note evidences of hyperplasia as in Fig. 14. Hematoxylin and eosin. Oil immersion.

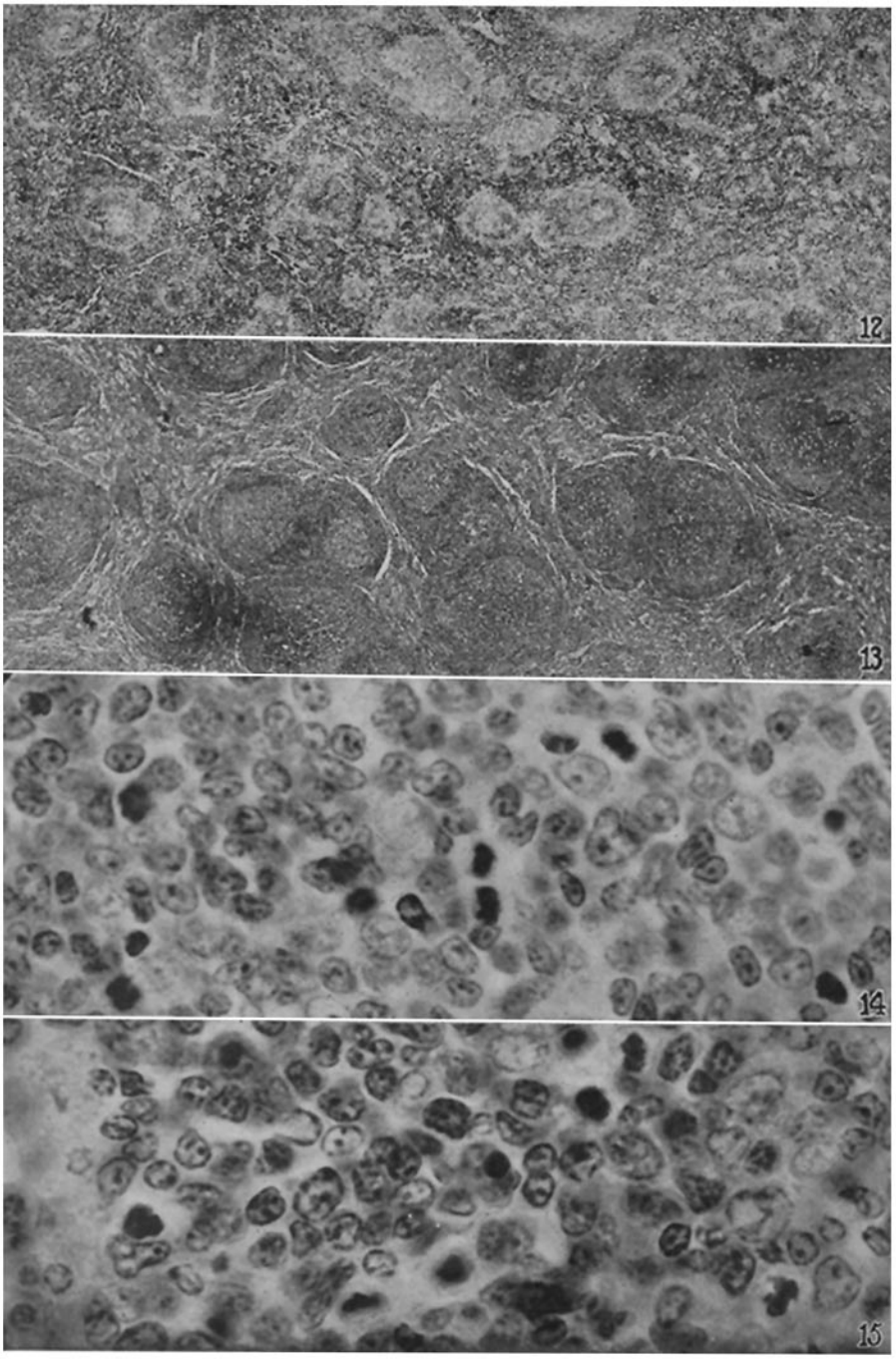


(Wiseman: Age of lymphocytes in peripheral blood)



Photographed by Louis Schmidt

(Wiseman: Age of lymphocytes in peripheral blood)



Photographed by Louis Schmidt

(Wiseman: Age of lymphocytes in peripheral blood)