AUTORADIOGRAPHIC STUDIES ON THE IMMUNE RESPONSE

I. The Kinetics of Plasma Cell Proliferation*

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Since the early experiments of Fagraeus (1), the role of plasma cells in antibody formation has been extensively studied and reviewed (2–7). Plasma cells have been found to contain (8–15) and produce (16–23) antibody, and apparently are the chief mediators of a humoral immune response. After the second injection of an antigen, far more plasma cells appear than after the first; this appears to be the main reason for the characteristic booster serum antibody response (18). Burnet and Fenner (2) first suggested that the secondary response might represent the proliferation of cells specifically altered by the primary immunization. Since the development of the clonal selection theory of acquired immunity (6, 24–27), there has been renewed interest in the nature of the plasma cell ancestor, the carrier of specific immunological memory.

The present experiments have used autoradiographic labeling techniques to study this question. They are predicated on the uptake of tritiated (H^3-) thymidine as an index of desoxyribonucleic acid (DNA) synthesis (28–30), and the validity of our conclusions depends on a number of premises which are widely held, but still the subject of active discussion (31–33). The premises most directly relevant to our experiments are: (*a*) the tritium label on thymidine is stable and does not exchange; nor does thymine base exchange after incorporation into DNA; (*b*) DNA itself is metabolically stable and turnover is insignificant in resting mammalian cells; (*c*) H^3-thymidine injected intravenously is rapidly distributed throughout the body, and either promptly incorporated into DNA or eliminated; (*d*) thymidine or H^3-thymidine does not affect the normal behavior of the cells that have taken it up. This last premise has recently been challenged (34).

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Several cell types have been named as likely plasma cell precursors, namely: (*a*) the small lymphocyte (4, 6); (*b*) a primitive lymphocyte (1, 11); (*c*) the reticular cell (2); (*d*) the macrophage or some other histiocytic cell (1, 35); (*e*) the few plasma cells seen during a primary response. These cell types differ markedly in the incidence of labeling shortly after the injection of H^3-thymidine (36–39); for example, after 1 hour, large lymphocytes are labeled in about 80 per cent of cases, but small lymphocytes rarely or never (37). In our experiments, the radioactive marker was introduced into animals primarily immunized against *Salmonella* flagella some weeks previously. Following removal of the tracer from the circulation, a secondary response was initiated, and animals were killed at intervals thereafter. Under these circumstances, whatever change in the proliferative pattern the secondary antigenic stimulation may have produced, the descendents of cells labeled prior to stimulation should have been labeled, and of cells unlabeled prior to stimulation, unlabeled. The results to be reported favor the hypothesis that all plasma cells are the result of recent cell division and originate from primitive cells dividing continuously *before* the second injection of antigen,—most probably large lymphocytes.

Materials and Methods

Animals.—Wistar albino rats supplied by a commercial breeder and not systematically inbred were used. They weighed 200 to 250 gm at the time of original injection, and were fed on Purina mouse chow and tap water.

Antigen and Immunization.—The antigen used was semipurified flagella from *Salmonella adelaide* (40, 21); for primary immunization, each rat received 25 μg into each hind foot-pad. 4 to 40 weeks later, some rats received a similar pair of injections as a secondary stimulus.

Isotope Injection.—H^3-thymidine samples of different specific activities (360 μc/μM; 1.9 mc/μM; 3.0 mc/μM) were obtained from Schwarz BioResearch, Inc., Mount Vernon, New York. Each rat received a single intravenous dose of from 0.1 to 8.0 μc/gm body weight. The animals were killed at intervals; those held for 2 hours or longer received 80 μmoles of H^1-thymidine 1 to 2 hours after the H^3-thymidine, in an attempt to dilute remaining pools of unincorporated tracer; those held for 24 hours or longer received daily intraperitoneal injections of 80 μmoles of H^1-thymidine to minimize reutilization of the tracer.

Preparation of Autoradiographs.—Rats were exsanguinated under ether anesthesia and the popliteal lymph nodes which drained the injection site were removed, pooled, weighed and covered with one to two drops of fetal calf serum. The cells were teased out with needles; smears were prepared on gelatin-coated slides, dried rapidly in air, fixed in 1 per cent acetic acid–90 per cent methanol overnight (36), and rinsed in running tap water for 30 minutes. Autoradiographs were then prepared according to Pelc's (41) technique, using Eastman Kodak AR 10 stripping film and exposure times of 1 to 130 days (usually 20 to 60 days). The slides were then stained with Giemsa at 16°–18°C., allowed to dry, and examined under oil-immersion at 1250-fold magnification. After development, the background grain count was frequently below one grain/1000 μ^2; slides with background >1 grain/50 μ^2 were discarded. To adopt an objective criterion of labeling applicable to all acceptable slides, cells with 3 or more grains overlying the nucleus were considered labeled, and those with 0 to 2 grains unlabeled. In fact, because of the doses of isotope and exposure times used, and the low back-

grounds, few cells with 1 to 10 grains were encountered, and labeling was generally quite unequivocal.

Histological Classification of Lymphoid Cells.—The cells were classified according to criteria previously described (19, 42, 43). Eight cell types frequently encountered were: (*a*) large lymphocytes (>10 μ nuclear diameter), this category includes cells referred to by others as hemocytoblasts, lymphoblasts, undifferentiated stem cells, and reticular lymphocytes (35); (*b*) reticular cells, with ovoid nuclei showing reticular chromatin pattern, and delicate fibrillary cytoplasm; (*c*) medium lymphocytes (7 to 9 μ nuclear diameter); (*d*) small lymphocytes (4 to 6 μ nuclear diameter); (*e*) plasmablasts, cells resembling large lymphocytes but having somewhat eccentric nuclei and basophilic cytoplasms. This cell has also been called the transitional cell (1) or simply primitive antibody-forming cell (4). It is difficult to distinguish from other primitive lymphoid cells, particularly when the surrounding cells are shielded from view and the observer does not know whether he is dealing with an immunized or an unimmunized animal. So, for most purposes we have pooled this category and the category of large lymphocytes; (*f*) immature plasma cells, (nuclear diameter 8 to 9 μ) with eccentric nuclei, bulky basophilic cytoplasm and perinuclear clear zone; (*g*) mature plasma cells; (*h*) smeared cells, amorphous pink smudges representing cells broken during smearing and drying. Other less frequent cell types included mast cells, macrophages and other histiocytes, eosinophils and neutrophils. Erythrocytes were occasionally seen but not scored.

Scoring of Smears.—The larger cells tended to occur more frequently along the edges of the smears. Differential cell counts were performed by scanning systematically across the middle section of each smear from edge to edge. 2000 to 4000 cells from each rat were differentially classified. Frequently this did not include a sufficient number of the rarer cell types; in such cases, the edges of the smear were scanned until 50 to 200 of each cell type had been classified as labeled or unlabeled. For determination of mean grain counts of blast cells (large lymphocytes and plasmablasts), 50 to 100 cells were scored. To obtain an "adjusted mean grain count," actual mean counts were normalized to a 30 day exposure time and 1 μc/gm isotope dose by multiplying the actual counts by (30/exposure time in days) $\times$ (1/isotope dose in μc/gm). Justification of this adjustment follows below.

Serum Antibody Titrations.—The titers of *Salmonella*-immobilizing antibody in serum samples were determined as previously described (40, 21). If an end point occurred at a serum dilution of 1:10, the titer was termed 1; of 1:20, 2; of 1:40, 3; and so forth.

RESULTS

Plan of the Experiments.—In all experiments, rats were killed at intervals following a single injection of H^3-thymidine. Three parameters which affected the grain counts of labeled cells could be varied, namely the amount of H^3-thymidine given, the specific activity of H^3-thymidine, and the autoradiographic exposure period. We minimized variations in the amount of thymidine given, but varied the dose of radioactivity and exposure time widely; obviously animals killed at longer periods after isotope injection, many of whose cells underwent a number of mitotic divisions between the isotope injection and the time of killing, needed larger doses of radioactivity, or longer exposure times, or both to give a readily scorable number of grains over each labeled cell. Preliminary study established what doses and times were needed to give at least 10 grains over nearly every labeled cell, and in the experiments to be reported, few, if any, cells would have been scored as negative because their content of tritium had been diluted below the threshold of detectability by repeated mitoses. With rare exceptions, the tracer dose varied from 0.25 to 8.0 μc/gm and the exposure time from 20 to 60 days. H^1-thymidine was given to all animals held for longer than 2 hours, as described above. The experiment involved 5 groups of rats: 2 groups had received a primary antigenic stimulus 4 weeks prior to the H^3-thymidine; of these 1 received a secondary stimulus 2

hours after the H^3-thymidine ("secondary response" group), the other (control) group received no antigen ("resting primarily immunized" group); 2 groups had received a primary antigenic stimulus 40 weeks prior to the H^3-thymidine; of these, 1 received a secondary stimulus 2 hours after the H^3-thymidine ("long memory secondary" group) and the other (control) group received no secondary antigen ("resting primarily immunized" group long memory experiment). Finally, the 5th group had received no antigen at any time ("unstimulated" group). It should be noted that the secondary stimulus was given after all the H^3-thymidine had been incorporated into DNA or eliminated; no H^3-thymidine was available to dividing cells *during* the actual secondary response.

The data obtained from examination of autoradiographs of popliteal lymph node smears from these rats have been expressed in 3 ways: the incidence of labeled cells is recorded graphically (*a*) as a proportion of the number of cells counted (Text-figs. 1, 2, 5, 6) or (*b*) corrected (Text-figs. 3, 4, 7, 8) for the weight of the lymph nodes, which hypertrophied after secondary antigenic stimulation (Table I). To arrive at the approximate total number of labeled cells in

TABLE I

*Wet Weights of Popliteal Lymph Nodes from Immunized Rats, (mg)**

Days after isotope injection	Resting primarily immunized rats	Mean	Secondarily immunized rats‡	Mean
0	11, 16, 17, 18, 19	16	—	—
1	12, 16	14	34, 25	30
2	10, 20	15	39, 71	55
3	16, 16, 17	16	48, 59, 77	61
4	15, 15	15	70, 40	55
5	—	—	36, 50	43
6	—	—	49	49

* Pooled weight of both nodes.

‡ Secondary antigenic stimulation 2 hours after isotope injection.

each popliteal lymph node pair, we used the assumption that 1 mg (wet weight) of node represented 10^6 cells; (*c*) the percentage labeling of each category of cells is recorded (Table II).

Plasma Cell and Lymphocyte Proliferation in Resting, Primarily Immunized Animals.—In rats killed 2 hours after the H^3-thymidine injections, only a small number of cells, namely some large and medium lymphocytes, a few smeared cells, and a very occasional plasmablast and immature plasma cell were labeled (Table II, Text-figs. 1 to 4). In the absence of secondary stimulation, the proportion of labeled cells seen on subsequent days rose gradually. In this steady-state situation, there was no significant change in the differential cell count and a very little plasmacytopoiesis. Large lymphocytes, which had been labeled in 80 per cent of cases at 2 hours, were almost all labeled on subsequent days; the proportion and total number of labeled medium and small lymphocytes also increased so that by the 4th day 6.7 per cent of all cells were labeled. The results are consistent with the view that the primitive lymphocytes

TABLE II

Per Cent of Lymph Node Cells of Various Categories Labeled Following Tritiated Thymidine*

		Hrs. after tritiated thymidine						
		2	25	48	72	96	121	149
Resting primarily stimulated animals‡	Large lymphocytes and plasmablasts	82	92	100	100	100		
	Medium lymphocytes	13	60	54	60	51		
	Small lymphocytes	0	2.0	1.5	4.7	3.8		
	Immature plasma cells	50§	75	83	100§	80		
	Mature plasma cells	0	17	35	10	30		
	Smeared cells	3.0	3.6	7.9	6.2	12.4		
	Reticular cells	0	0	7	4	16		
	All cells	1.2	2.8	4.0	5.2	6.7		
	Number of rats used	5	2	2	3	2		
Animals secondarily stimulated at 4 weeks‖	Large lymphocytes and plasmablasts		99.5	100	100	99	100	100
	Medium lymphocytes		50	68	89	90	76	77
	Small lymphocytes		3.6	4.2	10.5	14.4	12.4	8.3
	Immature plasma cells		71	96	99	97	97	100
	Mature plasma cells		6	20	93	96	95	97
	Smeared cells		12	23	50	54	31	30
	Reticular cells		12	13	3	30§	50	16
	All cells		5.4	11.0	28.0	48	34.1	28
	Number of rats used		2	2	3	2	2	1
Animals secondarily stimulated at 40 weeks‖	Large lymphocytes and plasmablasts	72	78	99.7	99	100	97	
	Medium lymphocytes	13	43	80	79	92	79	
	Small lymphocytes	0	3.4	4.2	4.9	15	23	
	Immature plasma cells	67	100§	95	98	98	99	
	Mature plasma cells	0	8	28	72	93	97	
	Smeared cells	0.9	11	15	23	51	53	
	Reticular cells	0	8	0§	0§	23	31	
	All cells	0.5	7.1	10.8	14.5	34.5	43.2	
	Number of rats used	1	1	1	1	1	1	

* Each figure in the table represents the percentage of cells in that category with nuclear labeling. In general, at least 50 cells of each category were scored from each animal; small lymphocytes were so numerous that over 1000 were scored as routine.

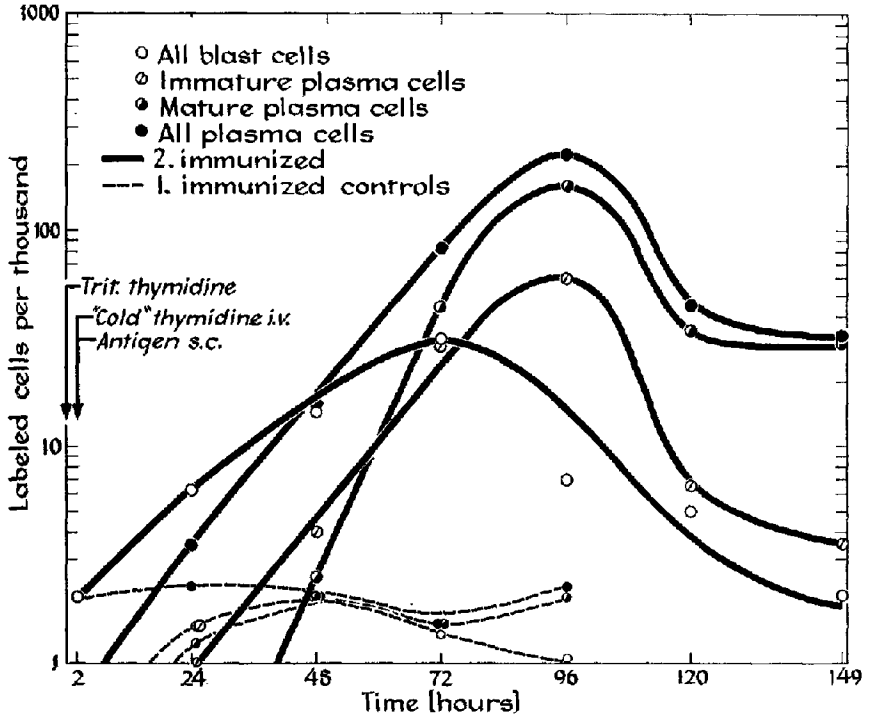
‡ Isotope injected 4 weeks after a single antigenic stimulus.

§ Too few cells of this category seen to give reliable percentage.

‖ Isotope injected 2 hours before secondary antigenic stimulus.

initially present had given rise to a sufficient number of their kind to maintain the *status quo*, and to medium and small lymphocytes. Of the few mature plasma cells seen, only a minority were labeled.

Plasma Cell and Lymphocyte Proliferation in Resting, Primarily Stimulated Animals: Long Memory Experiment.—Only a small number of such rats were available, so controls were studied only 2 hours and 1 day after H^3-thymidine.

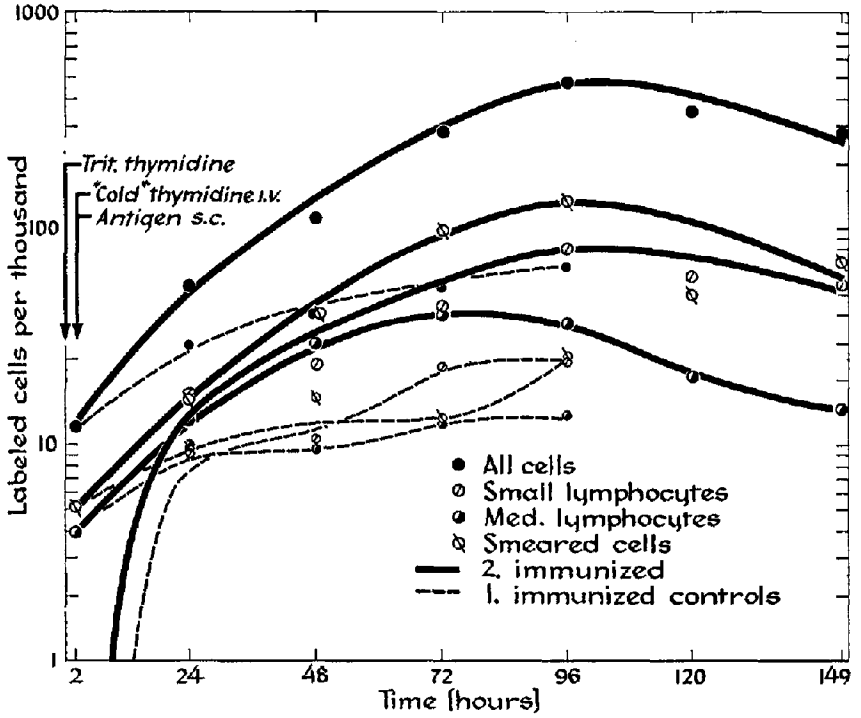


TEXT-FIG. 1. Proportion of labeled plasma cells in popliteal lymph nodes following tritiated thymidine. Secondary response.

Of all cells, only 0.5 per cent were labeled at 2 hours and 1.2 per cent at 1 day (Text-figs. 5 to 8). In these old rats, there was a smaller number of primitive cells and less lymphopoiesis than in the younger rats of the preceding group.

Plasma Cell and Lymphocyte Proliferation during the Secondary Response 4 Weeks after Primary Stimulation.—Secondary antigenic stimulation greatly altered the proliferation pattern of the lymph node cells. The proportion, and even more, the total number, of labeled large lymphocytes and plasmablasts ("all blasts") was increased already by 1 day (Text-figs. 1 and 3), and continued to rise until day 3, thereafter falling back to normal levels by the 6th day. The

number of plasma cells was first clearly above normal on the 2nd day, at which time mainly plasmablasts and immature plasma cells were seen; and rose exponentially until the 4th day, by which time mainly immature and mature plasma cells were present. At this stage there were about 400 times as many labeled plasma cells in the nodes as in resting controls; over 20 per cent of all cells were labeled plasma cells. Between the 2nd and the 4th day, the number

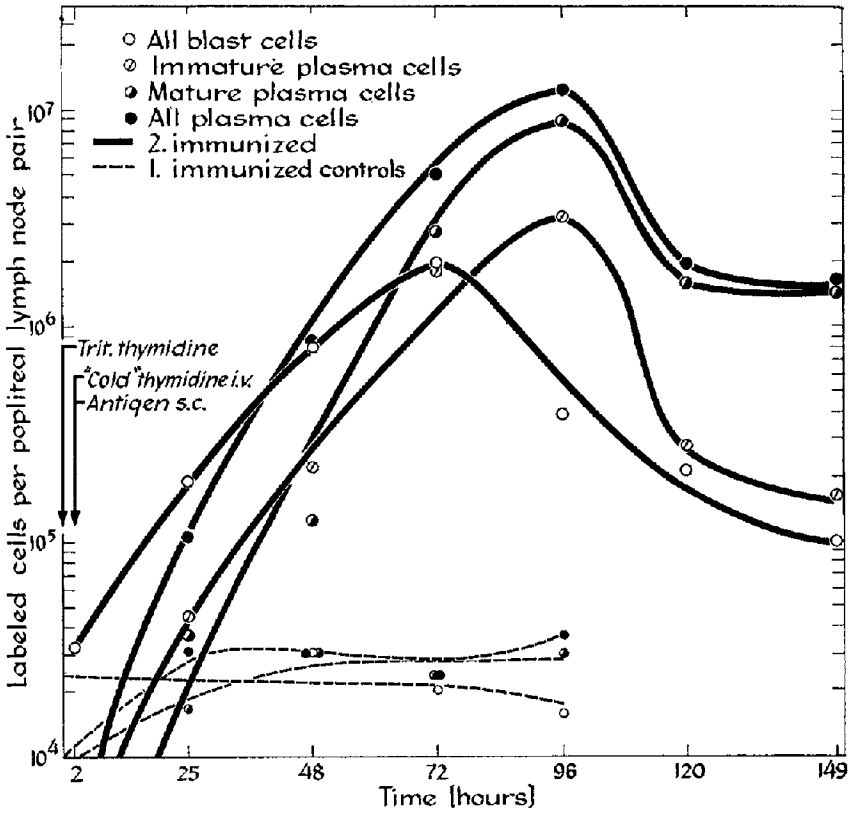


TEXT-FIG. 2. Proportion of labeled lymphocytes in popliteal lymph nodes following tritiated thymidine. Secondary response.

of labeled plasma cells doubled about every 12 hours. The proportion and absolute number of labeled lymphocytes also rose to a considerably greater extent than in control animals; on the 4th day, there were over 15 times as many labeled small lymphocytes in secondarily immunized nodes than in resting primarily immunized nodes; about half of all the cells in the node were labeled at that time. Between the 2nd and the 4th days, the total number of labeled cells doubled every 24 hours, and the number of labeled small lymphocytes every 30 hours. Few, if any, unlabeled plasma cells appeared as a result of secondary stimulation; over 95 per cent of all plasma cells seen after 3 days were labeled. After the 4th day, the number of labeled cells seen declined;

labeled plasma cells fell off rather sharply and labeled lymphocytes more gradually.

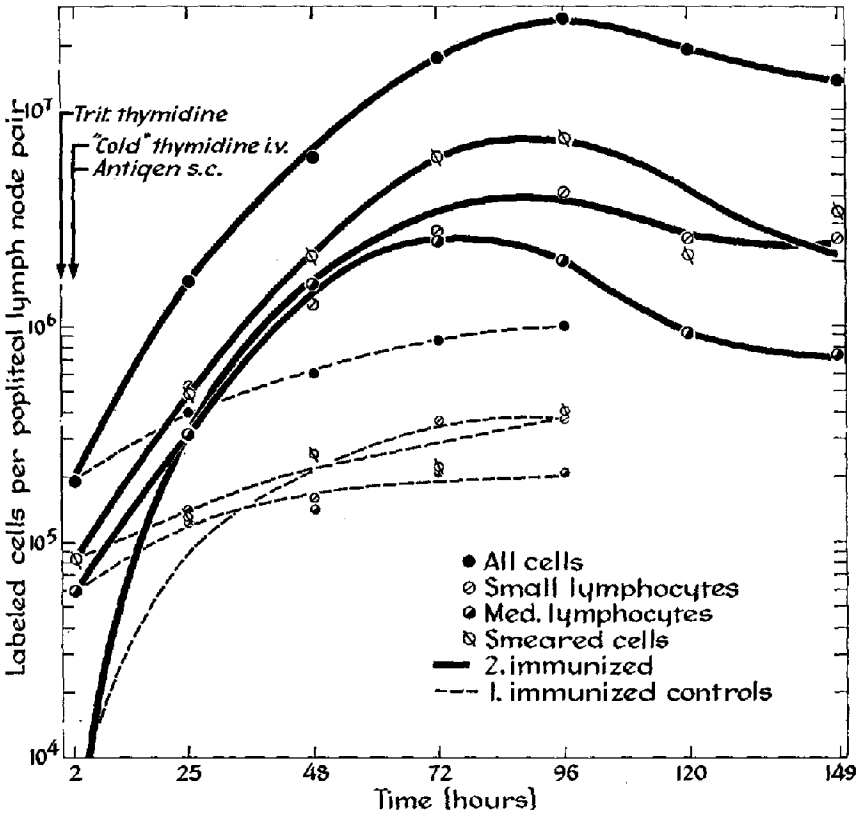
The incidence of labeled cells of some categories is not plotted graphically. Reticular cells generally comprised 0.3 to 0.8 per cent of the population, and were never labeled at 2 hours (Table II). There was no evident difference in



TEXT-FIG. 3. Total number of labeled plasma cells in popliteal lymph nodes following tritiated thymidine. Secondary response.

the number of labeled reticular cells seen in secondarily immunized and resting nodes. Mast cells and macrophages each comprised 0.1 to 0.2 per cent of the population. Mast cells were very rarely labeled; macrophage nuclei were never labeled at 2 hours and rarely thereafter; however, some macrophages already contained phagocytosed labeled nuclear debris at 2 hours. Some macrophages containing cytoplasmic labeling but no obvious nuclear debris, were seen at later stages. 1 day after H^3-thymidine and secondary antigenic stimulation neutrophil polymorphonuclear leucocytes comprised 1 to 2 per

cent of the lymph node cell population, and few of these were labeled. Apart from this, polymorphs were very rare in our nodes. All preparations contained 13 to 20 per cent of amorphous pink smudges presumably representing cells damaged during smearing or drying. Most of these were slightly larger than small lymphocytes and devoid of detectable blue cytoplasm. These were always

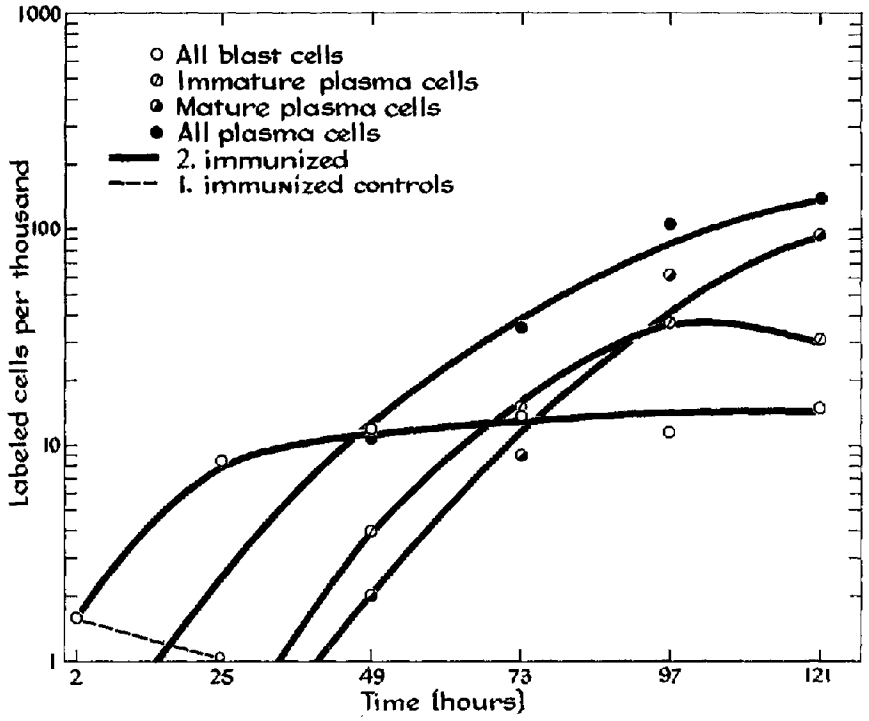


TEXT-FIG. 4. Total number of labeled lymphocytes in popliteal lymph nodes following tritiated thymidine. Secondary response.

labeled somewhat more frequently than small lymphocytes (Table II) and probably represent mainly young small lymphocytes. A few of the smeared cells were considerably larger, sometimes exhibiting clear holes; these were frequently labeled at 2 hours and thereafter, and may represent more primitive lymphocytes.

Plasma Cell and Lymphocyte Proliferation during the Secondary Response: Long Memory Experiment.—The proliferative pattern seen in rats secondarily stimulated 40 weeks after their primary injection was, in general, similar to

that seen in the short-term secondary response. The chief difference was that in the long memory experiment, fewer cells were labeled at 2 hours; possibly as a result, the curves showing the appearance of labeled plasma cells and lymphocytes during a secondary response (Text-figs. 5 to 8) lagged about a day behind the corresponding curves in the former experiment. In the long memory experiments, the absolute and relative numbers of labeled lymphocytes



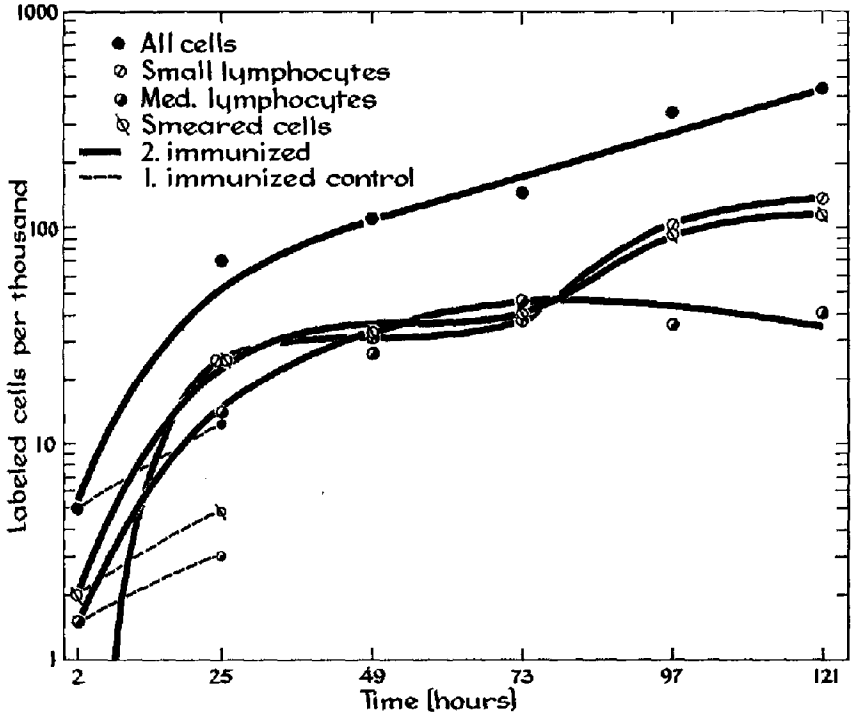
TEXT-FIG. 5. Proportion of labeled plasma cells in popliteal lymph nodes following tritiated thymidine. Long memory experiment.

and plasma cells were somewhat lower than in the short-term experiment. Again most of the plasma cells formed as a result of secondary stimulation were labeled.

Plasma Cell and Lymphocyte Proliferation in Unstimulated Animals.—Unstimulated animals showed a proliferative pattern similar to that of resting animals injected with H^3-thymidine 4 weeks after primary stimulation. This shows that the specific effect of primary antigenic stimulation on the proliferative pattern (if any) is no longer detectable after 4 weeks.

Mean Grain Counts of Primitive Cells.—There are a number of technical limitations in quantitative autoradiography which seemed to make an empirical

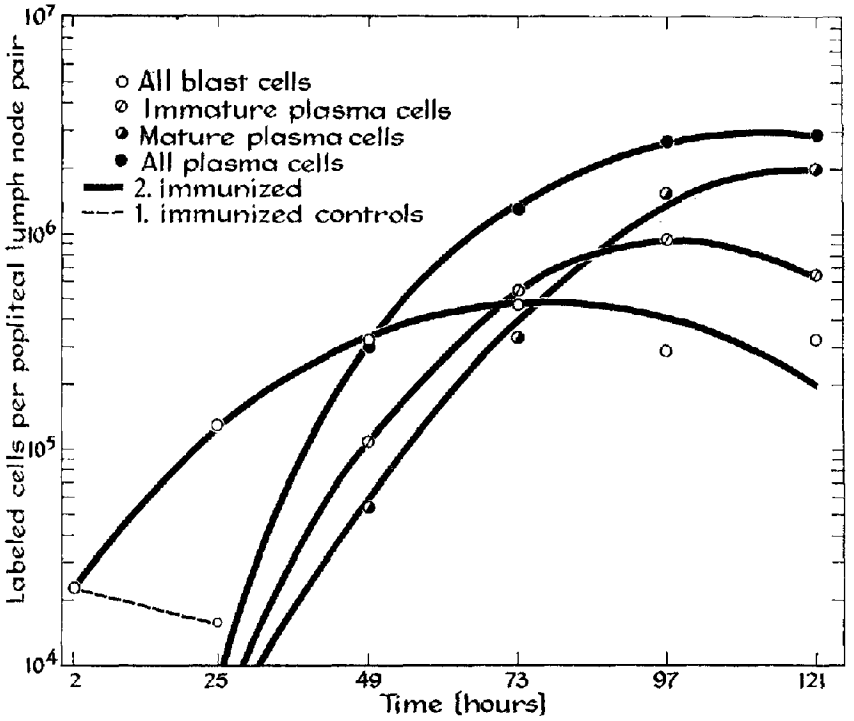
study of reproducibility of mean grain counts desirable. First, the half-life of tritium is 12.5 years and thus grain counts should be directly proportional to exposure times over the first months; however, latent image deletion might differentially affect autoradiographs exposed for varying periods. Secondly, the degree of flattening of the nucleus varies from cell to cell and from preparation to preparation—the more rapidly the smear dried, the more flattened the



TEXT-FIG. 6. Proportion of labeled lymphocytes in popliteal lymph nodes following tritiated thymidine. Long memory experiment.

cells, generally. As the average effective range of beta particles from tritium is only about 1 μ (44), considerable absorption of radiation could occur in rounded cells, thus reducing the radiation impinging on the emulsion. Thirdly, different batches of film may vary in sensitivity. Fourthly, there might be significant differences from animal to animal in the distribution of injected isotope throughout the body, or in the degree of extra- or intracellular dilution of isotope which might be expected. Thus an experiment was set up in which 3 rats were injected with 2.8×10^{-4} μmoles of H^3-thymidine/gm, 1 rat for each of the 3 different specific activities used. After 2 hours, the rats were killed and autoradiographs of mesenteric lymph node cell smears prepared. The

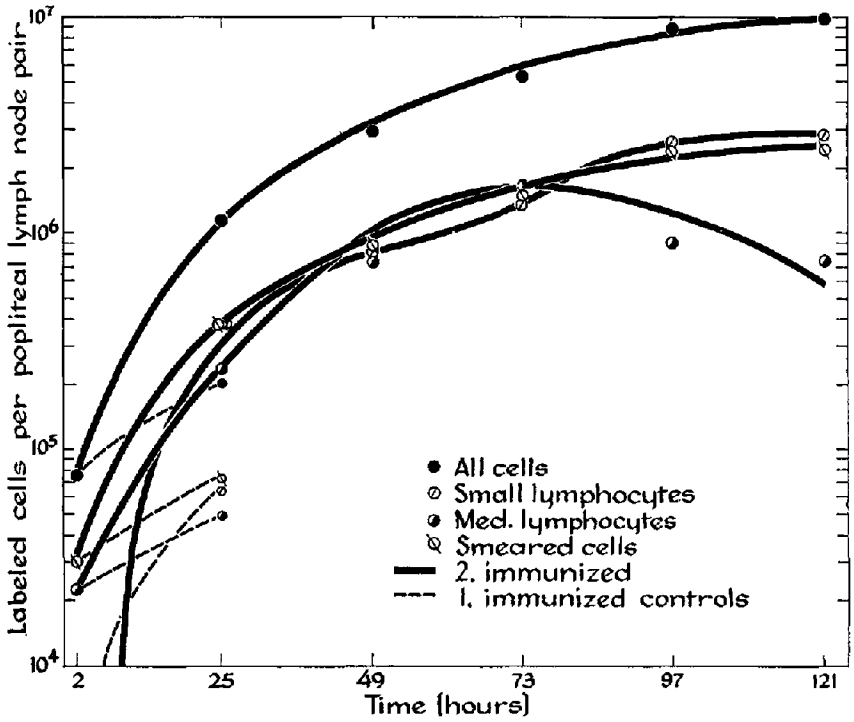
adjusted mean grain counts overlying blast cells were determined (Table III). Scatter was considerable, as can be seen from the large standard deviations. No significant differences either between replicate smears from the same animal or between animals were noted. Unfortunately mean grain counts could not be determined in slides exposed for longer than 33 days in this experiment, because fusion of individual grains became a problem. Experience with replicate slides



TEXT-FIG. 7. Total number of labeled plasma cells in popliteal lymph nodes following tritiated thymidine. Long memory experiment.

from our other experimental animals suggested that mean grain counts were proportional to exposure times for periods of up to 130 days. In general, we found it convenient to expose for between 20 and 60 days. Though the great variation amongst individual cells makes it difficult to reach a firm conclusion, we feel the grain count adjustment factors we have used were justified.

Text-fig. 9 shows the adjusted mean grain counts of blast cells from our experimental rats. The counts in secondarily immunized animals fell slightly more rapidly in the first 24 hours than in resting or unstimulated animals, but the differences were not statistically significant. The rates of fall of mean grain counts declined with time in both stimulated and unstimulated animals.



TEXT-FIG. 8. Total number of labeled lymphocytes in popliteal lymph nodes following tritiated thymidine. Long memory experiment.

TABLE III

Adjusted Mean Grain Counts of Blasts from Mesenteric Lymph Nodes

Specific activity of thymidine injected	Days of exposure at 4°C									
	1	2	4	7	11	13	18	21	26	33
360 μc/μM.....	—	340 ± 238	380 ± 231	479 ± 244	425 ± 242	432 ± 220	383 ± 218	393 ± 184	381 ± 206	411 ± 177
1·9 mc/μM.....	506 ± 237	583 ± 211	542 ± 65	547 ± 230	468 ± 201	430 ± 267				
3·0 mc/μM.....	455 ± 237	521 ± 250	478 ± 201	587 ± 434	475 ± 271	546 ± 366				

The adjusted mean grain counts of blasts from secondarily immunized animals were halved about every 7 hours on the 1st day and about every 15 hours on the 2nd day. The grain counts of cells other than blasts varied even more widely and means were not determined.

Serum Antibody Titers.—The *Salmonella*-immobilizing titers of serum samples taken at the time of killing of our rats are given in Table IV. Considerable

antibody was present in resting stimulated animals, even 40 weeks after primary immunization, indicating that some antibody formation must have continued in these animals for many months. No detectable rise in serum antibody titer occurred within the first 3 days after immunization; on the 4th day, 1 secondarily immunized rat had a definitely raised titer; by the 5th and 6th day all rats secondarily immunized at 4 weeks had markedly raised titers. This is consistent with our extensive experience with this antigen. Rats secondarily immunized at 40 weeks did not develop raised antibody titers in the first 5 days; it should be noted that in them, plasmacytopoiesis seemed to lag about 1 day behind that in rats immunized secondarily at 4 weeks.

TABLE IV

Serum Salmonella-Immobilizing Antibody Titers of Immunized Rats

			Titer (log to base 2)	Mean
Rats secondarily immunized at 4 weeks	Resting		5, 5, 5, 6, 7, 8, 8, 8, 9, 9, 9, 10, 10, 10	8
	Days after secondary stimulation	1	6, 8	7
		2	8, 9	8½
		3	6, 9, 10	8
		4	9, 12	10½
		5	13½, 13	13
		6	15	
Rats secondarily immunized at 40 weeks	Resting		6, 9	7½
	Days after secondary stimulation	1	8	
		2	9	
		3	8	
		4	7	
		5	7	

DISCUSSION

Nearly every plasma cell called into being during a secondary response is labeled if an injection of tritiated thymidine is given shortly before the secondary stimulus (Table II, Text-figs. 1, 3, 5, 7). If thymidine is incorporated only during DNA synthesis, and if DNA synthesis in somatic mammalian cells normally indicates impending mitosis (33, 36, 44), nearly every plasma cell must have resulted from recent cell division; this conclusion would eliminate the alternative suggestion that plasma cells arise purely by differentiation from some other cell types without intervening DNA synthesis and division.

A more subtle but equally important point emerges when we consider the conditions of isotope injection. A single, brief pulse of H^3-thymidine was given shortly *before* secondary stimulation; thus the plasma cells must have arisen from amongst the few cells already capable of synthesizing DNA in the resting

node (Table II, Text-fig. 1). The H^3-thymidine, given 2 hours before antigen, was probably completely removed from the circulation in about 10 minutes (36, 44); to dilute out any radioactive DNA precursors still possibly present in intracellular pools, we gave a large dose of H^1-thymidine immediately prior to secondary antigenic stimulation. H^3-thymidine was thus not available to dividing cells at any time during the secondary response. We postulate, with one reservation to be discussed below, that the only way in which plasma cells could have acquired nuclear labeling was by being the derivatives of cells initially labeled 2 hours *before* secondary stimulation. This would exclude from consideration as plasma cell progenitors those cell types which do not synthesize DNA in resting nodes: small lymphocytes, macrophages, mature plasma cells, and reticular cells. We may then consider the cell types which do show some DNA synthesis in resting nodes: a fraction of the medium lymphocytes and smeared cells; and the two categories of blast cells, 82 per cent of which are labeled 2 hours after H^3-thymidine injection. Typical plasmablasts are rare in resting nodes and thus it seems likely that the large lymphocyte is the chief ancestor of plasmablasts and plasma cells.

The frequency with which large lymphocytes are labeled by a short pulse of H^3-thymidine means that the period of DNA synthesis must occupy most of interphase. The proportion of blasts showing labeling at 2 hours (82 per cent in resting animals 4 weeks after primary stimulation, 72 per cent in resting animals 40 weeks after primary stimulation) is significantly lower than the proportion of blasts labeled at 2 days and thereafter which approaches 100 per cent in both resting and secondarily immunized animals (Table II). What becomes of the 20 per cent or so of unlabeled blasts? Possibly only those blasts synthesizing DNA continuously are capable of having progeny still morphologically blasts 2 days later. Perhaps the others have already embarked irrevocably on a course of differentiation towards the medium and small lymphocyte and are thus soon lost from the population of blasts.

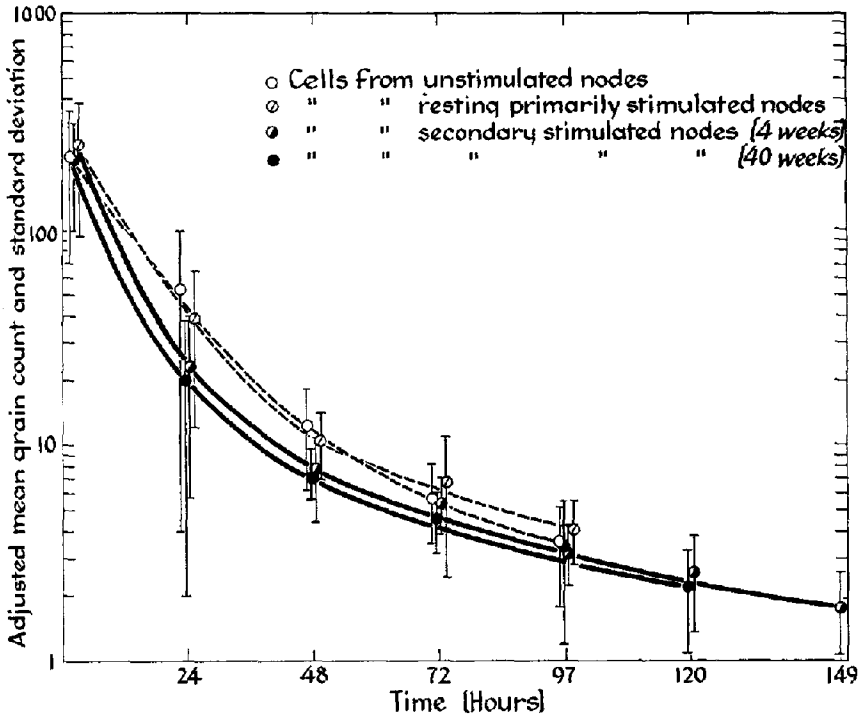
In resting nodes, with a constant number of cells of each type, the mitoses of large lymphocytes must be counterbalanced by an equal number of losses from this population, either through differentiation, presumably mainly to the most numerous cell type, the small lymphocyte, or through cell death. That some DNA-synthesizing cells die is clear from the presence of labeled nuclear debris in macrophages only 2 hours after H^3-thymidine injection, an observation also stressed by Cronkite *et al.* (33). In any case, only half the daughters of mitotic divisions of large lymphocytes remain large lymphocytes in resting nodes. A secondary antigenic stimulus apparently constitutes a signal for some of the rapidly dividing cells to engage in plasmacytopoiesis, and during the next 3 days, the primitive pool is enlarged. This may or may not be accompanied by an increased mitotic rate; it is sufficient that both progeny of many mitoses amongst blasts remain primitive and themselves have primitive progeny for

some generations. In the first 3 days the blasts also differentiate, showing increasingly basophilic cytoplasm and eccentric nuclei. The end result is an exponential increase of typical plasma cells with a doubling time of about 12 hours, and a peak level 4 days after secondary stimulation. There is little or no lag between the antigen injection and the initiation of these changes; increased numbers of blasts are seen already at 1 day (Text-figs. 1 and 3); more observations during the first 24 hours would certainly be useful. The apparent lag of 1 to 2 days in the appearance of increased numbers of labeled plasma cells presumably reflects the time taken for the differentiation process. The lag in the rise of serum antibody titer is longer. This finding is difficult to interpret because we have no idea how important a contribution the popliteal nodes make to the total antibody formed in the body. There may be a delay in the distribution of antigen to other, perhaps more important, antibody-forming sites. Moreover, the absolute number of plasma cells seen in the first few days is small; their contribution to increased antibody synthesis may be obscured because of residual antibody formation in the resting animal and crude assay technique. Finally, there may be a delay between the appearance of plasma cells and the release of antibody; we shall present evidence against this alternative in the accompanying paper. The stimulus for primitive cells to engage in plasmacytopoiesis in a secondary response evidently begins to wane after 3 days. The number of blasts seen thereafter soon approaches normal levels, and the number of labeled immature and mature plasma cells also falls off after 4 to 5 days (Text-figs. 1, 3, 5, 7). Secondary antigenic stimulation is also followed by increased lymphopoiesis (Text-figs. 2, 4, 6, 8), maintained for at least 4 days. This may be due in part to the presence in our flagella preparation of significant quantities of O antigen or endotoxin (21), which is known to cause lymphoid hyperplasia (46).

Any attempt to determine the kinetics of cellular proliferation in lymph nodes is complicated by the fact that we are not dealing with a closed system. Lymphocytes enter and leave *via* the lymphatics, and apparently *via* the blood stream as well (45). The cellular content of a node at any time is therefore the net result of a complex dynamic equilibrium. Our results suggest considerable migration of cells into popliteal lymph nodes during the first 2 days after a secondary antigenic stimulus. Cell proliferation alone could not have accounted for the observed weight increases (Table I, Text-Figs. 4 and 8).

Apparently a population of primitive cells sensitive to antigen is maintained for a long time after primary immunization. 40 weeks after a single stimulus, we were able to obtain a brisk plasma cell response (Table II, Text-figs. 5 and 7) with a secondary injection. Again, the labeling of plasma cells showed them to be derived from precursor cells rapidly dividing already in the resting nodes. Whether the primitive cells capable of responding at 40 weeks were the direct lineal descendants of those capable of responding at 4 weeks is not known as yet.

One reservation in accepting the above interpretations must be mentioned. Our argument depends on the condition that plasma cells could only have been labeled by virtue of their descent from cells labeled in the resting node by the single pulse of H^3-thymidine. However, some of the DNA-synthesizing cells in the body may have short life spans and may have died during the course of our study; it is possible that their DNA or DNA breakdown products may have then become available for incorporation into dividing cells. Other workers feel

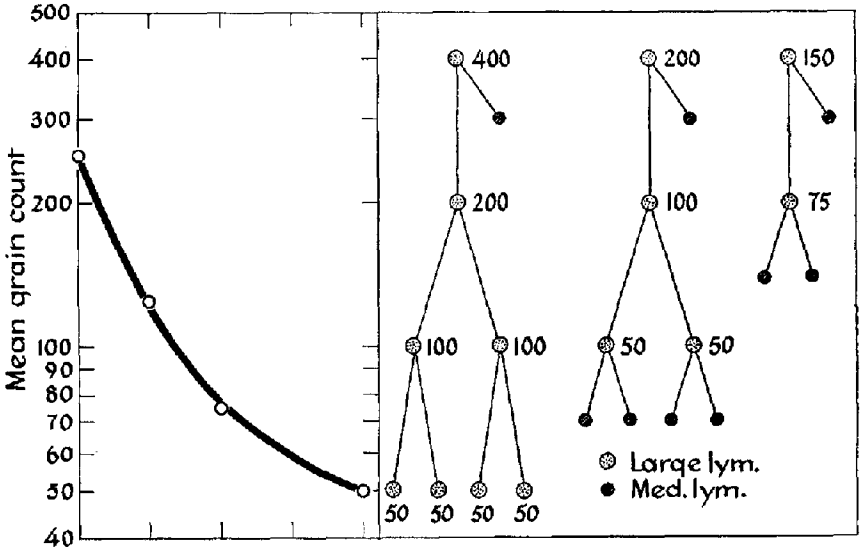


TEXT-FIG. 9. Mean grain count over blast cells in autoradiographs of popliteal lymph node smears.

that this is not a likely source of errors in interpretation (32). We have attempted to reduce reutilization by giving the rats daily injections of H^3-thymidine; it seems unlikely that reutilization could have given the high adjusted mean grain counts seen in the first 2 days, when the pattern of proliferation and differentiation was already well established.

The kinetics of the fall in mean grain count of blast cells (Text-fig. 9) are of interest, though detailed interpretation is made difficult by the large scatter of grain counts. If small molecular tritium-labeled compounds are removed from the cells by fixation and washing, and the observed grains really reflect tritium in DNA, radioactivity should be evenly distributed to both daughter

cells during mitosis; amongst a homogeneous population of cells, the time taken for the mean grain count to be halved should give the generation time. Unfortunately, large lymphocytes are not a homogeneous population; they vary considerably in size and appearance, and incorporate H^3-thymidine at widely differing rates. In resting primarily stimulated and unstimulated rats, the adjusted mean grain counts of blasts fell from over 200 to under 50 in the 1st day, suggesting a mean generation time of less than 12 hours. Subsequently, the rate of fall declined; after the 3rd day, counts were halved only every 36 hours. At first sight this suggests an over-all slowing of the mitotic rate; but



TEXT-FIG. 10. Possible explanation for decline in rate of fall in mean grain counts of blast cells in a steady-state situation.

in these groups this is impossible as we are dealing with a steady-state. One hypothesis to explain the kinetics is that the cells synthesizing DNA at the most rapid rate, and therefore with the highest grain counts, are the most primitive and have progeny remaining members of the blast category for some time; and that the cells synthesizing DNA more slowly are closer to their eventual destiny as medium and small lymphocytes, being thus soon lost to the population. An oversimplified model of this hypothesis is presented in Text-fig. 10. A second possibility is that reutilization of labeled DNA breakdown products takes place to an increasing extent, as discussed above. Decision between these and other possible explanations awaits the development of methods which can analyze the behavior of individual clones. In secondarily immunized rats, the counts fall even more rapidly in the 1st day, suggesting a possible increase in mitotic rate of some blasts; but the values are not statistically significantly

different from those in resting rats. Subsequently, the kinetics of grain dilution are very similar in all groups.

Three questions important to immunological theory are: (*a*) how does a given cell acquire the information to synthesize a given antibody? (*b*) how is it induced to express this information and embark on massive protein synthesis? (*c*) what are the mechanisms ensuring long lived immunity? Our studies have some relevance to the last two questions. Only cells continuously synthesizing DNA in resting nodes are capable of being induced; a single stimulus causes a self-limited burst of clonal expansion and differentiation to the plasma cell form in inducible cells. Long lived immunity may depend on the persistence of a continuously proliferating stem line of inducible cells, which may be maintained indefinitely subject only to random drift. If the immunological information were ever transferred to a resting cell, such as the small lymphocyte, one would have to postulate spontaneous reversions to the blast form in resting nodes; small lymphocytes as such do not seem to be inducible. If our formulation is correct, it suggests that the structures or compounds responsible for immunological memory must also be self-replicating. Unless trapping mechanisms exist, non-replicating entities such as antigen templates, would surely have been diluted out during the 500 or more mitotic divisions in the stem line in our long term experiments.

SUMMARY

The origin and growth kinetics of plasma cells have been investigated using autoradiographic labeling techniques. Rats immunized once with *Salmonella* flagella were given a single pulse of H^3-thymidine 4 or 40 weeks later. 2 hours after the tracer injection, they received a secondary antigenic stimulus. When animals were sacrificed immediately only certain cells from the resting primarily immunized lymph nodes, notably large and medium lymphocytes, were labeled. Subsequent to secondary stimulation, animals were killed at intervals; nearly all the plasma cells formed within the next 5 to 6 days were labeled. They must thus have been the progeny of cells already capable of synthesizing DNA in resting nodes, most probably of large lymphocytes. Plasmacytopoiesis began with little or no lag following secondary immunization, and the number of labeled plasma cells rose exponentially between the 2nd and 4th day, with a doubling time of about 12 hours. Studies of mean grain counts of primitive cells also suggested that the generation time of plasmablasts was 12 hours or less. The hypothesis was proposed that immunological memory depended on the persistence, following primary stimulation, of a continuously dividing stem line of primitive lymphocytes, reactive at all times to further antigenic stimulation.

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EXPLANATION OF PLATE 22

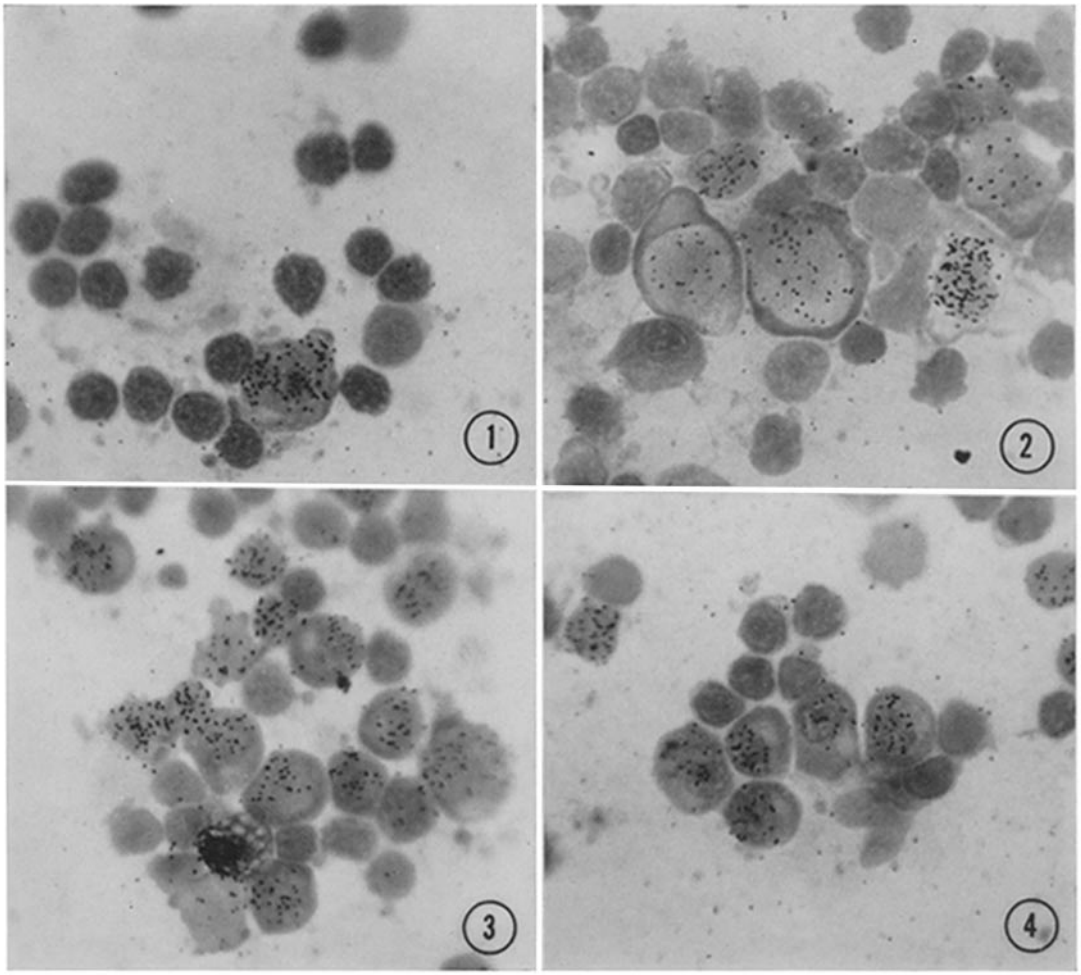
Autoradiographs of lymph node smears were prepared at various times after a single brief pulse of H^3-thymidine. 2 hours after the isotope, a secondary antigenic stimulus was given. Rats were killed at intervals: the times cited below refer to the period allowed to elapse between isotope injection and killing. × 2400.

FIG. 1. 2 hours. Note the labeled large lymphocyte and the unlabeled small lymphocytes.

FIG. 2. 2 days. Note the labeled plasmablasts, an unlabeled mature plasma cell, and an occasional labeled small lymphocyte.

FIG. 3. 4 days. Note the many labeled cells, including immature and mature plasma cells and small lymphocytes. A Russell-body plasma cell is very heavily labeled.

FIG. 4. 6 days. Note that the plasma cells now look quite mature, and are all labeled. Some labeled small lymphocytes are also present.



(Nossal and Mäkelä: Plasma cell proliferation)