SELECTIVE DNA SYNTHESIS BY CELLS SPECIFICALLY LOCALIZING IN RESPONSE TO XENOGENEIC ERYTHROCYTES*

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One of the basic effects of antigens on lymphocytes is to stimulate DNA synthesis and cell division. This effect is readily demonstrable in vitro (1–3) and in vivo (4–7). Thymus "independent" antigens may stimulate DNA synthesis by B lymphocytes only (8, 9) or by both T and B lymphocytes (10, 11), while thymus-dependent antigens typically stimulate DNA synthesis by both T and B cells (12, 13), with the response of the T cells generally preceding that of the B cells (5, 13). While some of this accelerated DNA synthesis reflects the activity of specific clones of lymphocytes with surface receptors for the antigen selected (14–17), much of it also seems to reflect the activity of cells with no intrinsic specificity for the antigen (15, 16, 18–25). These latter cells presumably are responding to mitogenic factor (18, 19), possibly to other T cell products (20–25) and possibly to other substances whose sources are not presently defined. This background of nonspecific stimulation has made it very difficult to study the antigen-induced DNA synthesis of specifically reacting cells directly.

We have recently described a system whereby it is possible to concentrate recirculating long-lived lymphocytes of a particular specificity by secondary antigenic stimulation (15, 16). Unfortunately this secondary stimulation also results in the nonspecific trapping of many other long-lived recirculating lymphocytes in the same lymph nodes (15, 16). The present studies, which were undertaken to try to refine this system, are based on the following two observations: first, most rapidly dividing lymphoid cells do not ordinarily recirculate (26) and therefore have little tendency to accumulate in antigenically stimulated lymph nodes (27, Emeson, Thursh, and Noble, unpublished observation), and, second, cells with specific reactivity for an antigen seem to rapidly synthesize DNA at very specific times after antigenic stimulation (28, 29). It was hoped that labeling cells for a very brief period after antigenic stimulation might make it possible to define conditions where specifically reactive cells were labeling much more rapidly than other recirculating

lymphocytes, and where most of the nonspecifically labeling cells were non-recirculating and therefore not available for localization in the secondarily stimulated lymph nodes.

Materials and Methods

Animals.—Genetically inbred female CBA/J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine.

Immunization.—Groups of CBA/J mice not less than 8 wk of age were immunized to either sheep red blood cells (SRBC)[1] (Grand Island Biological Co., Grand Island, N.Y.) or chicken RBC (CRBC) (courtesy of Doctors L. W. Schierman and R. A. McBride) as previously described (16). In one experiment the immunizing dose of SRBC was reduced to 10×10^8 cells and that of CRBC to 5×10^8 cells, divided between the intravenous and intracutaneous routes. Similar CBA/J mice were immunized with *Brucella abortus* (BA) stained ring test antigen (courtesy of Dr. D. E. Pietz, U. S. Dept. of Agriculture, Animal and Plant Health Inspection Service, Ames, Iowa) using 2×10^8 organisms, half intravenously and half divided among the four foot pads.

Labeling of Lymphoid Cells.—In vivo labeling of lymphoid cells was essentially as previously described (15, 16) using the same total dose of [^{3}H] or [^{14}C]thymidine. The labeling periods were, however, reduced from 12 days to 6–8 days, and the total number of injections of isotope in some cases was reduced from 24 to 18.

In vitro labeling with uridine or thymidine was performed by incubating cells with isotopically labeled compounds (New England Nuclear Corp., Boston, Mass.) for 45 min at 37°C. Each milliliter of the incubation mixture contained 1×10^8 living lymphoid cells, prepared as a single cell suspension as previously described (15), 25 μCi of ^{3}H or 5 μCi of ^{14}C-labeled nucleic acid precursor, 0.15 ml IPT gamma globulin free fetal calf serum (previously heated to 56°C for 30 min), 100 U of penicillin and 100 μg streptomycin in tissue culture medium-199 (TC-199) (Grand Island Biological Corp.). After incubation the cells were washed three times in 50 ml volumes of Hanks' balanced salt solution (HBSS) and adoptively transferred to syngeneic female mice as previously described (15).

Challenge.—The recipient mice were challenged with 0.05 ml of a 40% suspension of SRBC or CRBC, or with 0.05 ml of saline containing 1×10^8 BA organisms, divided between one front and one rear paw.

Experimental Design.—In the first series of experiments, donor mice were immunized with CRBC or SRBC, then 3, 10, 17, or 31 days later started on a 6–8 day course of [^{3}H] or [^{14}C]thymidine. 3 wk after the last injection of isotope the donors were sacrificed and their cells were pooled and injected into groups of syngeneic recipients. These recipients were then challenged with CRBC in the right front and rear paws and SRBC in the left front and rear paws and sacrificed 3 days later. The draining lymph nodes (axillary, brachial, popliteal, and iliac) were removed and segregated as to side for radiochemical analysis. Two groups of mice immunized 61 days before sacrifice (one with CRBC, the other with SRBC) were not given any isotope in vivo; the cells of these mice were labeled in vitro with [^{3}H] or [^{14}C]uridine just before adoptive transfer.

In the second group of experiments, mice immunized to CRBC or SRBC were sacrificed at various times after immunization and their cells incubated in vitro with ^{3}H- or ^{14}C-labeled uridine or thymidine, then adoptively transferred. In one experiment unlabeled BA immune cells were mixed with the pool of labeled SRBC and CRBC immune cells before transfer.

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[1] *Abbreviations used in this paper:* BA, *Brucella abortus* stained ring test antigen; CRBC, chicken red blood cells; dpm, disintegrations per minute; HBSS, Hanks' balanced salt solution; R, normalized ^{3}H (^{3}H/^{14}C); SE, standard error of the mean; SLC, specifically localizing cells; SRBC, sheep red blood cells.

In some experiments all recipients were challenged with both SRBC and CRBC as above. In other experiments, mice were challenged with SRBC only or with CRBC only, using BA as a nonspecific (with respect to the labeled cells) challenge on the contralateral side.

Preparation of Samples and Counting Procedures.—Lymph nodes were dried and defatted with acetone and dissolved in 1 N NaOH as previously described (15). In some experiments where in vitro labeling with uridine had been performed DNA and RNA were separated by the Schmidt-Thannhauser method (30) and then counted separately. The clear aqueous alkaline samples were suspended in a dioxane Cab-O-Sil (Packard Instrument Company, Downers Grove, Ill.) cocktail as previously described (15). ^{3}H and ^{14}C were counted simultaneously (31) in a Packard liquid scintillation spectrometer (model 3375) (Packard Instrument Company).

Treatment of Data.—The treatment of data was as previously described (15, 16). Very briefly, this involves calculating the normalized ^{3}H (R) for each set of stimulated lymph nodes (dpm ^{3}H/dpm ^{14}C), and comparing the two sets of draining lymph nodes of each recipient mouse by dividing the R of one set of nodes by the R of the other set of nodes. For convenience, the ratio was arranged so that values greater than 1.00 indicate an immunologically specific accumulation of the appropriately labeled cells in each set of nodes. In all the experiments described in this communication, CRBC immune cells were labeled with ^{3}H and SRBC immune cells with ^{14}C.

Where individual recipients were challenged with either CRBC or SRBC it is possible to estimate the absolute amount of radioactivity (in dpm) carried to the stimulated lymph nodes by specifically localizing cells (SLC) from the formulae:

$$[^3H]SLC = [^3H]CRBC - [^{14}C]CRBC \ (R_{BA}), \ \text{and}$$
$$[^{14}C]SLC = [^{14}C]SRBC - [^3H]SRBC \ (1/R_{BA}),$$
$$\text{where} \ R_{BA} = [^3H]BA/[^{14}C]BA.$$

These formulae are based on the assumption that in the absence of specific localization the R values of different lymph nodes (other then mesenteric) of a single recipient will be very nearly equal. We have considerable data indicating that this assumption is valid (16, Thursh and Emeson, unpublished observation).

Statistical Methods.—The data from recipients challenged with both CRBC and SRBC was analyzed as previously described (15, 16) using the Student t test and the sign test. In the sign test, when the ratio R_{CRBC}/R_{SRBC} was greater than 1.00 the animal was given a plus sign; when the ratio was less than 1.00 it was given a minus sign. Values of 1.00 ± 0.01 were considered equal and were not included in the calculation. The probability of obtaining the observed number of plus signs or more than the observed number of plus signs from any group of recipients was calculated from the binomial distribution (32) with $P = 0.5$ in the expression $\binom{n}{r}P^r q^{n-r}$ (33).

The data from recipients challenged with either CRBC or SRBC plus the indifferent antigen BA were analyzed in the same way, the terms being arranged so that values greater than 1.00 indicate immunologically specific localization.

RESULTS

The results of experiments involving in vivo labeling of anti-CRBC SLC with [^{3}H]thymidine and anti-SRBC SLC with [^{14}C]thymidine are summarized in Table I. These data clearly show that SLC are synthesizing DNA more rapidly than other recirculating cells during the first postimmunization period tested (days 3–8 or 3–10) and probably during the second period (days 10–15) as well. It is equally obvious that from the third postimmunization week on,

TABLE I

Specific Localization of Long-Lived Lymphocytes Labeled In Vivo with $[^3H]$ or $[^{14}C]Thymidine$

Group	No. of mice	Days of labeling	No. of mice positive* Total no.–no. equal	P	Mean R_{CRBC}/R_{SRBC}	P
1	6	3–10	6/6	<0.02	1.11	<0.01
2	5	3–8	4/4	<0.10	1.06	<0.01
3	5	10–15	4/5	NS	1.05	<0.05
4	6	17–22	0/0	NS	1.00	NS
5	5	17–24	3/4	NS	1.02	NS
6	6	31–28	1/1	NS	1.00	NS
7	6	61‡	3/3	NS	1.02	NS

* In the sign test data which are neither + nor — are not included in the calculation of P. R_{CRBC}/R_{SRBC} ratios of 1.00 ± 0.01 are considered equal.

‡ Donor cells labeled in vitro with $[^3H]$ (anti-CRBC) or $[^{14}C]uridine$ (anti-SRBC).

SLC synthesize DNA no more rapidly than other recirculating lymphocytes. The total amount of isotope incorporated by SLC during the first 6–8 days is comparable to that which we have previously reported for SLC labeled with the same total dose of radioisotope over a 2 wk period (days 3–15) (16). There is a suggestion of some specific localization of cells labeled in vitro with $[^3H]$ or $[^{14}C]uridine$ (no effort was made to separate labeled RNA from labeled DNA in this particular experiment) on day 61, but the differences in the R_{CRBC}/R_{SRBC} in this group are so small (mean 1.02) that we hesitate to attach much significance to them.

The observation that it is possible to label the DNA of SLC over a much shorter period than we had previously thought possible, led us to the idea that SLC might be more selectively labeled in vitro than in vivo. In the first pre-liminary experiment, uridine rather than thymidine was used as the labeled nucleic acid precursor, because we felt there might be even greater selectivity of RNA synthesis by SLC during this time. The results of this experiment were somewhat surprising in that while the RNA of SLC did show slight selectivity of labeling (5/5 mice in this group had R_{CRBC}/R_{SRBC} values in the direction of immunological specificity, with a mean value of 1.03) on postimmunization day 3, there was much more striking selectivity in the labeling of the DNA of SLC (mean R_{CRBC}/R_{SRBC} value of 1.28) on the same day. A much greater degree of specificity could be demonstrated when thymidine rather than uri-dine was used as a precursor. Under these conditions, R_{CRBC}/R_{SRBC} ratios greater than 3.0 were observed for the first time, thus indicating that it would now be possible to study the response to one antigen at a time and thereby to express results in absolue terms (the actual dpm's of ^3H or ^{14}C carried to lymph nodes by SLC) rather than in relative terms (R_{CRBC}/R_{SRBC}) such as had been necessary up to now.

The results of the first experiment using this procedure are summarized in Table II. In this experiment all 15 recipients received a single pool of [³H]thymidine labeled anti-CRBC cells, [¹⁴C]thymidine labeled anti-SRBC cells and unlabeled anti-BA cells. Recipients 1–5 were challenged with SRBC on one side and BA on the other, recipients 6–10 with CRBC and BA, and recipients 11–15 with CRBC and SRBC. These data resemble those in Table I and those previously reported (16) in that all 15 recipients showed differences in the

TABLE II

Specific Localization of Lymphoid Cells Labeled In Vitro with [³H] or [¹⁴C]Thymidine

Animal	Right sided lymph nodes			Left sided lymph nodes			Ratio	SLC	
	dpm ³H	dpm ¹⁴C	R	dpm ³H	dpm ¹⁴C	R		dpm	%
	CRBC challenged			BA challenged			R_{CRBC}/R_{BA}		
1	3,198.3	82.1	39.0	3,863.1	142.1	27.2	1.44	965.2 (³H)	30.2
2	3,844.5	95.4	40.3	3,865.5	150.4	25.7	1.57	1,392.7 (³H)	36.2
3	3,665.0	92.6	39.6	3,907.9	140.9	27.7	1.43	1,100.0 (³H)	30.0
4	4,544.2	95.1	47.8	5,218.8	178.3	29.3	1.63	1,757.8 (³H)	38.7
5	3,837.4	97.3	39.4	4,385.8	156.4	28.1	1.40	1,103.3 (³H)	28.8
	3,817.9	92.5	41.2	4,248.2	153.6	27.6	1.49	1,263.8 (³H)	32.8
	BA challenged			SRBC challenged			R_{BA}/R_{SRBC}		
6	3,263.0	119.5	27.3	2,367.2	157.0	15.1	1.81	70.3 (¹⁴C)	44.8
7	4,357.2	147.5	28.9	2,364.0	185.6	12.7	2.27	103.8 (¹⁴C)	55.9
8	3,727.8	136.4	27.3	2,311.5	232.3	9.9	2.76	147.6 (¹⁴C)	63.6
9	3,506.7	128.9	27.2	2,002.8	176.6	11.3	2.41	103.0 (¹⁴C)	58.3
10	2,810.2	102.9	27.3	2,422.7	181.4	13.4	2.04	92.7 (¹⁴C)	51.1
	3,513.0	127.0	27.6	2,293.6	186.6	12.5	2.26	103.5 (¹⁴C)	54.7
	CRBC challenged			SRBC challenged			R_{CRBC}/R_{SRBC}		
11	4,392.2	96.3	45.6	2,199.2	190.5	11.5	3.97	—	—
12	4,365.5	94.8	46.1	2,463.1	155.5	15.8	2.92	—	—
13	4,350.8	109.6	39.7	2,428.1	193.6	12.5	3.18	—	—
14	4,423.2	88.5	50.0	2,226.9	193.0	11.5	4.34	—	—
15	5,180.3	108.3	47.8	3,139.4	202.4	15.5	3.08	—	—
	4,542.4	99.5	45.8	2,491.3	187.0	13.4	3.50	—	—

R_{CRBC}/R_{BA}, R_{BA}/R_{SRBC}, and R_{CRBC}/R_{SRBC} ratios of the appropriately challenged lymph nodes consistent with an immunologically specific partitioning of the transferred lymphocytes according to the antigens used for challenge. The data in Table II differ markedly, however, in the magnitude of the differences observed (a mean R_{CRBC}/R_{SRBC} value of 1.14 is the largest one ever observed using in vivo labeled long-lived cells). The R_{BA}/R_{SRBC} of mice challenged with SRBC + BA (2.26) plus the R_{CRBC}/R_{BA} of mice challenged with CRBC + BA (1.49) approximates this R_{CRBC}/R_{SRBC} value of 3.50 seen in mice challenged with both SRBC and CRBC simultaneously, suggesting that the SLC of the CRBC and SRBC immune populations are partitioning inde-

pendently of each other. This is also demonstrated by looking at the same data from a different point of view; it is possible to abolish the evidence of immunologically specific partitioning of the two populations of labeled lymphocytes in animals 11–15 by subtracting the percentage SLC_{CRBC} [(0.328) ^3H] from the right sided lymph nodes and the percentage SLC_{SRBC} [(0.547) ^{14}C] from the left sided lymph nodes.

In a second series of experiments using this procedure, summarized in Table III and in Figs. 1 and 2, we have studied the in vitro incorporation

TABLE III

Specific Localization of Lymphocytes Labeled In Vitro at Various Times After Primary Immunization

Group*	Antigens for challenge‡	Day	R_{CRBC} or R_{SRBC} ± SE		R_{BA} ± SE		$\dfrac{R_{CRBC}}{R_{BA}}$	$\dfrac{R_{BA}}{R_{SRBC}}$
1	CRBC	2	27.8 ± 1.20	(CRBC)	19.1 ± 0.24		1.46	—
2	SRBC	2	15.3 ± 0.69	(SRBC)	19.3 ± 0.29		—	1.26
3	CRBC	3	21.0 ± 0.61	(CRBC)	11.5 ± 0.38		1.83	—
4	SRBC	3	8.47 ± 0.45	(SRBC)	13.6 ± 0.31		—	1.61
5	CRBC	4	20.1 ± 0.90	(CRBC)	14.8 ± 0.39		1.36	—
6	SRBC	4	12.9 ± 0.74	(SRBC)	14.6 ± 0.35		—	1.13
7	CRBC	5	12.5 ± 0.50	(CRBC)	8.81 ± 0.13		1.42	—
8	SRBC	5	8.01 ± 0.11	(SRBC)	8.87 ± 0.23		—	1.11
9	CRBC	7	10.8 ± 0.33	(CRBC)	8.91 ± 0.13		1.21	—
10	SRBC	7	9.42 ± 0.19	(SRBC)	9.18 ± 0.07		—	0.97
11	CRBC	11	13.4 ± 0.30	(CRBC)	11.5 ± 0.29		1.17	—
12	SRBC	11	12.3 ± 0.27	(SRBC)	11.7 ± 0.17		—	0.95
13	CRBC	14	10.3 ± 0.37	(CRBC)	9.95 ± 0.30		1.04	—
14	SRBC	14	10.0 ± 0.26	(SRBC)	9.72 ± 0.23		—	0.97
15	CRBC	24	9.60 ± 0.26	(CRBC)	8.68 ± 0.04		1.11	—
16	SRBC	24	9.60 ± 0.26	(SRBC)	9.21 ± 0.17		—	0.96

* There are four recipients in each group. Paired groups (e.g. 1 and 2) received a single pool of [^3H]thymidine labeled CRBC immune cells and [^{14}C]thymidine labeled SRBC immune cells.

‡ All mice challenged with antigen listed on right side and with BA on left side.

of [^3H] and [^{14}C]thymidine into the DNA of SLC as a function of the time after immunization. In these experiments we did not feel it necessary to adoptively immunize the recipients to BA in order to generate enough nonspecific trapping of recirculating lymphocytes in the contralateral lymph nodes for a suitable radiochemical control. In Fig. 1 the total ^3H, the [^3H]SLC and the [^3H]SLC/total ^3H × 100 (percent SLC) of the CRBC challenged lymph nodes has been plotted against the time (post primary immunization) of labeling, and in Fig. 2 the total ^{14}C, the [^{14}C]SLC and the [^{14}C]SLC/total ^{14}C × 100 (percent SLC) of the SRBC stimulated lymph nodes has been similarly plotted. In both cases there is an increased incorporation of radioactive thymidine
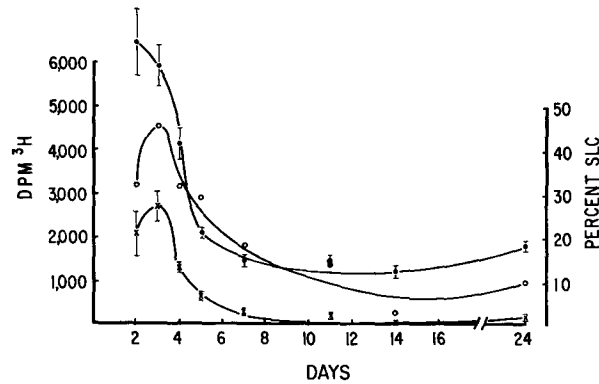
FIG. 1. The total accumulation of ^{3}H-labeled lymphoid cells (●) ± SE and the accumulation of ^{3}H-labeled anti-CRBC SLC (X) ± SE in CRBC challenged lymph nodes, along with the percent SLC (O), calculated by dividing [^{3}H]SLC by total ^{3}H and multiplying by 100.

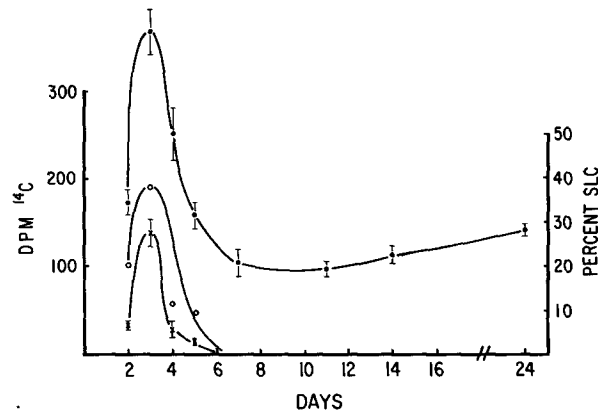FIG. 2. The total accumulation of ^{14}C-labeled lymphoid cells (●) ± SE and the accumulation of ^{14}C-labeled anti-SRBC SLC (X) ± SE in SRBC challenged lymph nodes, along with the percent SLC (O), calculated by dividing [^{14}C]SLC by total ^{14}C and multiplying by 100.

into lymph node seeking cells generally as early as 48 h after primary immunization, and over and above this a preferential incorporation of radioactive thymidine into the DNA of SLC at the same time. In both cases (Figs. 1 and 2) the rate of DNA synthesis by SLC, both absolute (dpm) and relative (percentage of the total count) seems to peak on day 3, at which time 45.3% of the ^{3}H in the CRBC challenged lymph nodes and 38.1% of the ^{14}C in the SRBC challenged lymph nodes has been carried there by SLC. In other experiments, 32.8–43.4% of the ^{3}H and 51.7–54.7% of the ^{14}C carried to the specifically challenged lymph nodes by cells labeled on postimmunization

day 3 could be attributed to SLC, and the general shapes of the curves (for the first 5 days) were very similar.

<center>DISCUSSION</center>

The present studies have shown that there are specific periods after primary immunization when SLC are synthesizing DNA very much more rapidly than other recirculating lymphocytes. During these periods a brief (45 min) in vitro exposure to radioactive thymidine results in a very highly selective labeling of these cells. When these selectively labeled SLC are transferred to syngeneic hosts and allowed to respond to local antigenic challenge, they can account for as much as 50% of the total observed radioactivity in a set of stimulated lymph nodes.

The present studies have also shown that SLC labeled with [^{3}H] or [^{14}C]uridine (which labels long-lived cells in a random way) at a time when SLC can be assumed to have reverted to the resting state (61 days post primary immunization) fail to show convincing evidence of specific localization. This probably relates to the fact that SLC numerically constitute only a small fraction of the total number of recirculating lymphocytes trapped in an antigenically stimulated lymph node. An additional factor could be a low intrinsic rate of RNA synthesis (34) in SLC belonging to the long-lived B lymphocyte population.

The original demonstration of an immunologically specific trapping of recirculating long-lived lymphocytes in antigenically stimulated lymph nodes (15, 16), while of considerable theoretical importance, has only limited practical application as a model in the study of cellular immunity, principally because of limitations in the degree of selectivity of labeling of SLC achieved. The procedure for in vivo labeling of SLC, which was based upon published methods for labeling long-lived lymphocytes in resting animals (35), resulted in an immunologically specific component in the partitioning of two populations of labeled lymphocytes in response to challenge with two antigens of only 10-15%, even using up to 80 μCi ^{14}C and 360 μCi ^{3}H per donor mouse. Obviously it would be extremely difficult to adapt this method to the study of one antigen at a time, where it was expected that the immunologically specific component would be only half as large. The large amounts of isotope needed to label long-lived cells effectively, and the long times needed to complete a single experiment, also limit the usefulness of this technique. The in vitro techniques described in this communication seem to overcome most of those difficulties. We believe that when the problems discussed below are more fully understood, in vitro labeling of SLC will make it possible to apply the model of immunologically specific lymphocyte trapping to studying the generation, dissemination and expression of immunological memory along with many other problems of cellular immunity more directly than has previously been possible.

In the present studies we see a rather sharp peak in the rate of DNA synthesis by SLC on the third postimmunization day, with a rapid decline in this rate over the next few days. In the SRBC immunized mice, no detectable selectivity of labeling of SLC is observed after day 5, while in the CRBC immunized mice, SLC seem to continue synthesizing DNA at an increased rate throughout the duration of the experiment (24 days). We suggest that this probably reflects differences in the relative doses of the two antigens used, though intrinsic qualitative differences in the response to CRBC as opposed to SRBC may also play a role (36). It is not entirely clear exactly when the increase in the rate of DNA synthesis by SLC begins, though the evidence now available suggests that this starts between 24 and 48 h after immunization.

It is interesting to note that the curve of preferential DNA synthesis by SLC in the SRBC immunized mice (Fig. 2) is almost identical to the curve for T cell mitosis in response to this same antigen reported by Davies et al. (5). DNA synthesis by antibody-forming cell precursors, which are of course B lymphocytes, has also been described as starting within 24–48 h of primary immunization (37). While we believe that many and perhaps most SLC are T lymphocytes, at present there is no direct proof of this available. There is on the other hand evidence that B cells are capable of specific localization (38), though under rather different conditions from those described here. We are presently exploring the effect of anti-θ on SLC to try to resolve this point.

We are not certain of the significance (if any) of the "reversed specificity" observed in *13/16* SRBC challenged recipients of cells labeled on days, 7, 11, 14, and 24 (Table III). It seems possible that this represents an immunological cross reaction whereby SRBC cause the trapping of some ^{3}H-labeled anti-CRBC cells, even though only very slight cross reaction between these two antigens has been described in other systems (39, 40). Other explanations are also possible. It could be postulated, for example, that there are different rates of reutilization of [^{3}H]thymidine (41) in the SRBC and BA stimulated lymph nodes, the excess [^{3}H]thymidine (over [^{14}C]thymidine) being available because of the greater cytotoxicity of the very low energy ^{3}H beta emission and the higher specific activity of the [^{3}H]thymidine used for labeling (28, 42, 43).

The much higher values of R_{CRBC}/R_{SRBC} obtained using in vitro (3.50) rather than in vivo (1.10) labeling are also very interesting. There are at least three possible explanations for this discrepancy, which we believe is mainly the result of differences in the time of labeling relative to the time of adoptive transfer. First is the possibility that many of the SLC labeling in vitro are differentiating "end cells" that die during the 3-wk waiting period after the in vivo labeling. A less likely possibility (in view of the relatively small amount of new radioactive thymidine incorporated by SLC after day 5) is that these cells continue to divide in vivo and thus dilute out their label on

this basis. Finally, it is possible that after postimmunization day 5 the rate of DNA synthesis by SLC relative to that of other recirculating cells declines to the point where there is dilution of the selectivity of labeling of SLC.

It is possible that at least part of the excess in vitro labeled DNA carried to lymph nodes by SLC represents "metabolic" DNA (44) associated with cellular differentiation which may have a more rapid rate of turnover (44) than the "genetic" DNA synthesized during the S phase of the cell cycle. This could account for the loss of label from SLC during the 3 wk waiting period after in vivo labeling just as well as differentiation and cell death postulated above. If this proves to be the case, the technique described here for concentrating the labeled DNA of cells differentiating in response to a single antigen (at the peak, 50% of the labeled DNA in challenged lymph nodes is associated with SLC) should prove to be a valuable one for defining and characterizing this type of DNA. Several investigators have previously attempted to demonstrate DNA's of this type in mouse plasma cell myelomas by differences in their hybridization with labeled specific RNA. Some have presented rather encouraging results (45), while others have failed to detect any evidence of gene amplification under these conditions (46). Having the unique DNA selectively labeled rather than the RNA would obviously provide a much more sensitive system for studying this question.

SUMMARY

The present studies have shown that cells capable of specific localization in response to challenge with CRBC or SRBC synthesize DNA very rapidly during the period from 2–5 days (peak 3 days) post primary immunization. This has been done by incubating the antigenically stimulated lymphoid cells with [^{3}H] or [^{14}C]thymidine in vitro for 45 min before adoptive transfer to syngeneic recipients. Specifically localizing cells (SLC) labeled in this way may ultimately account for up to 50% of the ^{3}H or ^{14}C present in a set of specifically challenged lymph nodes 3 days later. The data presented are consistent with the hypothesis that SLC numerically constitute only a very small fraction of the total number of recirculating lymphocytes trapped in antigenically stimulated lymph nodes, and that the demonstration of specific localization therefore depends upon selectively labeling these SLC relative to other recirculating cells. Attempts to selectively label the RNA of SLC with the precursor uridine have to date met with only very limited success.

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