*ENHANCEMENT OF IN VIVO INCORPORATION OF LABELED PRECURSORS INTO DNA AND TOTAL PROTEIN OF MOUSE LYMPH NODES AFTER ADMINISTRATION OF THYMIC EXTRACTS**

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Communicated by Alfred Gilman, February 18, 1965

In a study of the protein composition of lymphoid tissue, Roberts and White[1] in 1949 reported that a fraction isolated from calf thymus produced thymic hypertrophy when injected for a ten-day period into adult rats. Since this suggestion of a humoral role for the thymus in stimulating lymphocytopoiesis, considerable additional evidence[2–5] has appeared. This effect appears to have some relation to the role of the thymus in the acquisition of immunological competence after birth[6] or after whole body irradiation.[7] Investigation into the apparent lymphocytopoietic activity of thymic extracts has been hampered by the lack of a rapid assay method. Utilization of a peripheral blood lymphocytosis as assay of lymphocytopoiesis is not only tedious, but is subject to criticism on theoretical grounds.[8]

Estimation of the rate of lymphocyte proliferation by measurement of incorporation of isotopically labeled precursors appears to offer many advantages in the detection of lymphocytopoietic activity in various extracts of thymus glands. Previous work in this laboratory[9] has shown the usefulness of this approach in studying the effects of steroid hormones on lymphoid tissue.

In this communication we wish to report the enhanced *in vivo* incorporation of tritiated thymidine into DNA, and of glycine-2-C^{14} into total protein of mouse lymph nodes following injection of either calf, rat, or mouse thymic extracts. The data obtained utilizing labeled thymidine afford a basis for assessing the activity of thymic extracts in promoting lymphocyte proliferation.

Materials and Methods.—Frozen calf thymus, obtained from a local abattoir, fresh rat thymus, obtained from 180–240-gm male Sprague-Dawley rats, or thymus from groups of adult, CBA mice, was homogenized in 0.15 *M* NaCl or in 0.1 *M* phosphate-Versene buffer, pH 5.7 (tissue:solvent = 3:1) at 0–4°C. The homogenate was then centrifuged at 700 × *g* in an International refrigerated centrifuge at 0°C for 15 min and the supernatant then centrifuged at 105,000 × *g* in a Spinco model L ultracentrifuge for 1 hr. The clear supernatant obtained was used for assay. Some thymic extracts were prepared according to the method of De Somer and co-workers[4] by incubating minced thymus aseptically in Hank's solution for 48 hr with subsequent separation of the cell-free supernatant. All extracts were used immediately after preparation or stored at −20°C for up to three weeks. Extracts of rat spleen and lymph nodes were prepared with 0.1 *M* phosphate buffer as with thymus above.

Inbred male CBA mice, 55–70 days old, from a colony maintained in our laboratory were used for all assay experiments. Mice born on the same or on successive days were used together to make the experimental groups as homogeneous as possible.

Extracts were injected intraperitoneally into groups of animals at periods of 24–72 hr before sacrifice. Control groups were injected with buffer. Two hours before sacrifice, a pulse label of H^3-thymidine (3 mc/mmole, Schwarz BioResearch, New York), 10 or 15 μc per mouse, or glycine-2-C^{14} (12 mc/mmole, New England Nuclear Corp., Boston), 0.20 μc/gm body weight, was injected intraperitoneally. The mice were sacrificed by cervical fracture. Axillary and inguinal lymph nodes, thymii, and spleens were removed rapidly from each group of mice, weights of the respective pooled tissues recorded, and then placed in 0.15 *M* saline at 0–4°C.

In the experiments utilizing H^3-thymidine, the lymphoid organs were homogenized at 0–4°C

in 7% perchloric acid, using a chilled Potter-Elvehjem homogenizer. After centrifugation, the pellet was washed twice with cold perchloric acid, and three times with absolute ethanol. Nucleic acids were extracted with 2 ml of 7% perchloric acid at 70–80°C for 20 min. After cooling and centrifugation, 0.2-ml aliquots were pipetted into 15 ml of Bray's solution, neutralized with KOH, and counted in a Packard Tri-Carb scintillation counter. DNA content was measured by the method of Dische.[10]

In the experiments utilizing glycine-2-C^{14}, the mouse lymphoid tissue was homogenized in 10% trichloroacetic acid in the cold, and centrifuged in a Clay-Adams centrifuge. The resulting pellet was washed twice with trichloroacetic acid, twice with ethanol:ethyl ether, 1:1, and once with ethyl ether. The washed proteins were plated on preweighed filter paper disks on aluminum planchets; the latter were weighed again and counted in a Nuclear-Chicago gas-flow (Q-gas) counter. Protein concentrations were measured by the biuret method.

Results.—The thymic extracts did not contain DNA (negative diphenylamine reaction); the presence of RNA was indicated by a positive orcinol reaction, seen also with dialyzed extracts. Repeated freezing and thawing of extracts resulted in loss of lymphocytopoietic activity. Phosphate buffer (0.1 *M*, pH, 7.4) extracts of rat thymus showed no activity in the assay used. However, 0.1 *M* phosphate buffer extracts of pH 5.7 were active.

During the course of these studies it was found that subjecting the animals to undue stress, e.g., drafts, cold, excessive handling, poor water or food supply, either before or during the experimental period, resulted in markedly diminished precursor incorporation and little or no difference in incorporation between experimental and control groups. These findings may be attributed to augmented pituitary-adrenal cortical activity, with consequent effects on lymphoid tissue structure and function.[11]

Effect of extracts on lymphoid organ weights: Twenty-four hr following a single injection of thymic extract, no detectable alteration in lymphoid organ weight was noted in comparison with saline-injected controls. However, in 72-hr experiments using three daily injections of extract, a consistent and marked increase in lymph node weight resulted (Table 1). The weight of the spleen was usually increased in these experiments, while thymus weight was found to be decreased in many experiments. These findings were evident after injection of calf and rat[11a] thymic extracts (the latter prepared according to the method of De Somer and co-workers[4]). Injection of a similar quantity of bovine serum albumin caused a decrease in weight of lymph nodes.

TABLE 1

EFFECT OF INJECTION OF THYMIC EXTRACTS AND OF BOVINE SERUM ALBUMIN ON MOUSE LYMPH NODE WEIGHT

Type of extract*	No. of animals per experiment	Total protein per injection (mg)	No. of injections	Duration of experiment (hr)	Lymph node wt† (mg/gm body wt)	% Δ
0.15 *M* NaCl	6	0	3	72	1.07	—
Calf thymic extract	7	7.6	3	"	1.44	+34.6
0.15 *M* NaCl	7	0	3	"	0.92	—
Calf thymic extract	7	7.6	3	"	1.17	+27.2
0.15 *M* NaCl	4	0	3	"	0.97	—
BSA‡	4	5.0	3	"	0.77	−20.6
Hank's solution	6	0	3	"	0.81	—
DES§	7	0.68	3	"	1.02	+25.9

* Each injection in a volume of 0.5 ml.
† Pooled axillary and inguinal lymph nodes.
‡ BSA = bovine serum albumin.
§ DES = rat thymic extract prepared according to the method of De Somer and co-workers.[4]

TABLE 2

EFFECT OF INJECTION OF THYMIC EXTRACTS AND OF BOVINE SERUM ALBUMIN ON *in vivo* INCORPORATION OF GLYCINE-2-C^{14} INTO TOTAL PROTEIN OF MOUSE LYMPH NODES

Type of extract*	No. of animals per experiment	Total protein per injection (mg)	No. of injections	Duration of experiment (hr)	Counts/ min/mg lymph node protein	% Δ
0.15 *M* NaCl	6	0	3	72	34.8	—
Calf thymic extract	7	7.6	3	"	41.7	+20.3
0.15 *M* NaCl	7	0	3	"	31.4	—
Calf thymic extract	7	7.6	3	"	47.9	+52.6
0.15 *M* NaCl	4	0	3	"	36.9	—
BSA†	4	5.0	3	"	37.8	+2.4
Hank's solution	6	0	3	"	32.3	—
DES‡	7	0.68	3	"	36.8	+13.8

* Each injection in total volume of 0.5 ml.
† BSA = bovine serum albumin.
‡ Rat thymic extract prepared according to the method of De Somer and co-workers.[4]

Effect of extracts on incorporation of glycine-2-C^{14} into total protein of lymph nodes: In two experiments (Table 2) a pulse label of glycine-2-C^{14} following three daily injections of calf thymic extract (72-hr experiment) resulted in enhanced incorporation of isotope into lymph node protein above control values. In contrast, administration of bovine serum albumin, as another foreign protein, did not produce a significantly increased degree of incorporation. The rat thymic extract (De Somer) did not induce a large increase in glycine-2-C^{14} incorporation (13.8%). However, a marked increase in lymph node weight had occurred (Table 1), suggesting that the proliferative stimulus of the extract had already been translated into newly formed cells. Incorporation of glycine-2-C^{14} into thymus was often found to be decreased after injection of thymic extracts for 3 days, while enhancement of splenic incorporation was frequently found; however, these changes did not occur as consistently as did those in peripheral lymph nodes.

Effect of extracts on incorporation of H^3-thymidine into peripheral lymph nodes: In order to assess more precisely changes in *proliferative* activity of lymphoid tissue, the incorporation of H^3-thymidine into total nucleic acids was studied. Since thymidine is almost exclusively incorporated into DNA,[12] separation of DNA from

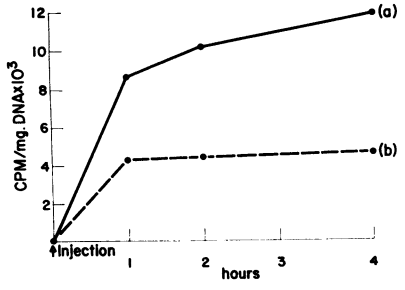

FIG. 1.—Time course of incorporation *in vivo* of H^3-thymidine (3.0 c/mmole, 15 μc per animal) into mouse lymphoid tissue. (*a*) Pooled axillary and inguinal lymph nodes; (*b*) thymus. Each point represents the mean of 4 animals.

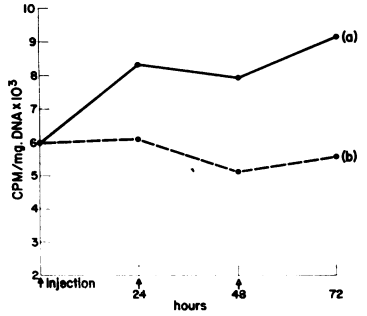

FIG. 2.—Incorporation *in vivo* of H^3-thymidine (3.0 c/mmole, 15 μc per animal) into mouse pooled axillary and inguinal lymph nodes after daily injections of (*a*) rat thymic extract, 0.5 ml (5.3 mg protein), and (*b*) 0.15 *M* NaCl, 0.5 ml. Each point represents the mean of 4 animals.

RNA was not considered necessary. Results are expressed as counts per minute per mg of DNA. Small differences in body weight of the groups of animals used were compensated for in the calculations in order to approximate dose of precursor per gram of body weight for all groups.

The time course of incorporation of H^3-thymidine into DNA was first studied. The data depicted in Figure 1 are in agreement with those of Nygaard and Potter.[13] The greater part of the incorporation into DNA occurred within the first 60 min after administration of precursor. A 2-hr pulse-label period was selected for subsequent studies.

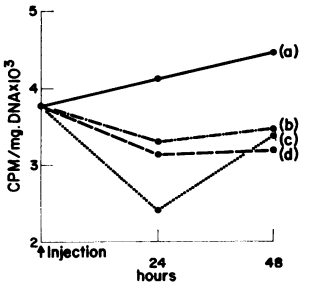

FIG. 3.—Incorporation *in vivo* of H^3-thymidine (3.0 c/mmole, 10 μc per animal) into mouse pooled axillary and inguinal lymph nodes after a single injection of (*a*) rat thymic extract, (*b*) rat spleen extract, (*c*) rat lymph node extract, (*d*) 0.15 *M* phosphate buffer, pH 5.7. Each point represents the mean of 6 mice each injected with 0.5 ml of extract containing 1.0 mg of protein or with 0.5 ml of buffer.

Enhancement of incorporation of H^3-thymidine into total nucleic acids (and therefore DNA) of lymph nodes by calf thymic extract after 72 hr (3 daily injections) was marked (Table 3); a 178 per cent increase was observed. The effect was apparent after 24 hr (1 injection), with a 45.4 per cent increase above control values. Extracts prepared from rat thymus also were active in enhancing incorporation of the precursor into DNA (Fig. 2 and Table 3), but extracts of lymph nodes and spleen did not affect thymidine incorporation (Fig. 3). Extracts of mouse thymus were also active in increasing H^3-thymidine incorporation into DNA (Table 3). In one experiment employing male mouse thymic tissue extracts, a single injection of 0.9 mg of protein in 0.3 ml of phosphate buffer resulted in 34.1 per cent enhancement of incorporation after 24 hr; in a second experiment using thymus from female mice, 26.8 and 19.0 per cent increases in incorporation were found, although the protein content of the extract was higher.

Discussion.—In 1944 Andreasen and Ottesen[14] reported the use of the rate of incorporation of a labeled precursor (P^{32}) as a measure of proliferative activity of various lymphoid organs. Hull and White[15] found a depression of incorporation of P^{32} into lymphocyte RNA after ACTH administration, while Fichtelius and coworkers[16] reported an enhancement of P^{32} incorporation into acid-insoluble phosphate of thymus and lymph nodes of rats after injection of pertussis vaccine, an agent known to produce a marked blood lymphocytosis.

Increased *in vivo* incorporation of tritiated thymidine into germinal center lymphocytes of mouse lymph nodes during an immune response to tetanus toxoid was found by Cottier and colleagues.[17] Their data suggest that the extent of incorporation of the precursor into lymphocyte DNA during pulse-labelexperiments *in vivo* is a valid estimate of proliferative activity of these cells.

Metcalf[18] in 1956 proposed the existence of a lymphocytosis stimulating factor elaborated by the thymus gland. The suggestion by Metcalf[3] and by Gregoire and Duchateau[2] that this factor may be secreted by the thymic epithelial cells has been supported by more recent electron microscopic studies,[19, 20] showing that these cells contain an extensive endoplasmic reticulum and numerous cytoplasmic inclusions. The assay system used by Metcalf was the production of lymphocytosis in neonatal

TABLE 3

EFFECT OF INJECTION OF EXTRACTS OF THYMUS ON *in vivo* INCORPORATION OF H^3-THYMIDINE INTO DNA OF MOUSE LYMPH NODES*

Type of extract†	No. of animals per experiment	Total protein per injection (mg)	Duration of experiment (hrs)	Counts/ min/mg lymph node DNA ($\times 10^3$)	% Δ
0.15 *M* NaCl	4	0	24	6.9	—
Calf thymic extract	4	6.2	24	9.6	+45.4
0.15 *M* NaCl‡	4	0	72	4.6	—
Calf thymus extract‡	4	6.2	72	12.8	+178
0.1 *M* Phosphate buffer (pH 5.7)	4	0	24	3.33	—
Rat thymic extract	4	1.9	24	4.70	+41.0
0.1 *M* Phosphate buffer (pH 5.7) 0.3 ml	3	0	24	3.84	—
Male mouse thymic extract 0.3 ml	4	0.9	24	5.15	+34.1
0.1 *M* Phosphate buffer (pH 5.7)	4	0	24	3.06	—
Female mouse thymic extract 0.4 ml	4	2.6	24	3.88	+26.8
Female mouse thymic extract	4	3.3	24	3.64	+19.0

* In the experiments represented by the first four lines of the table, 15 μc of H^3-thymidine were given each mouse. In the remaining experiments, 10 μc of H^3-thymidine were given each mouse.
Each animal received 1 injection, except as noted.
† Each injection in a total volume of 0.5 ml, except as noted.
‡ Three injections.

mice after injection of thymic extracts. However, he was not successful in stimulating lymphocytosis in intact adult mice by this procedure.[21] Using a different technique of extract preparation, De Somer and co-workers[4] recently reported production of blood lymphocytosis in adult mice after injection of thymic extract.

The data presented in this communication show that after 3 daily injections of heterologous, particle-free thymic extracts, significant increases in lymph node weight occur accompanied by increased incorporation of glycine-2-C^{14} into total protein and of H^3-thymidine into DNA of peripheral lymph nodes. It is apparent that the thymic extracts have induced increased proliferative activity in this lymphoid tissue. That the effect is due to the presence of a thymic lymphopoietic factor and not a result of an antigenic response to foreign protein is suggested by the lack of significant effect by extracts of rat lymph nodes and spleen, and of bovine serum albumin, on precursor incorporation into mouse lymph nodes. The stimulatory effect of isologous mouse thymus extract on H^3-thymidine incorporation lends further weight to this conclusion. The extract prepared according to the method of De Somer and co-workers[4] and shown by those investigators to repair the effects of neonatal thymectomy, was found also to enhance lymph node weight and incorporation of glycine-2-C^{14} into lymph nodes as well as to induce peripheral blood lymphocytosis.[11a]

Incorporation of H^3-thymidine into lymph node nucleic acids (DNA) was found to be increased approximately 40 per cent above control values 24 hr after injection of thymic extracts. This increase preceded definite weight changes which occurred later, 24–72 hr after start of administration of extract. It will be of interest to determine the cellular locus of the effect of the thymic preparation on lymphoid tissue by employing techniques of radioautography after H^3-thymidine incorporation. This approach should provide information regarding which class of lymphocytes is affected by the proliferative stimulus. Since the technique described in

this communication can rapidly assay lymphopoietic activity in tissue extracts, it should prove useful in further chemical fractionation of thymic extracts with the goal of isolation and characterization of the lymphocytopoietic factor. This project is currently in progress in our laboratory.

Summary and Conclusions.—Particle-free sodium chloride and acid phosphate buffer extracts of thymic tissue obtained from either mice, rats, or calves were injected into male CBA mice. After 24 hr, a significant increase was noted in pulse-label H^3-thymidine incorporation into the nucleic acids of peripheral lymph nodes as compared to control groups receiving injections of saline or buffer. After three daily injections of extracts (72 hr) further increments in H^3-thymidine incorporation into lymph nodes were observed. Increased pulse-label glycine-2-C^{14} incorporation into total protein and significantly increased lymph node weight were also noted at this time. Bovine serum albumin and extracts of rat lymph nodes and spleen did not produce similar effects. It is suggested that the effects of the thymic extracts on lymph node proliferation are due to the presence of a thymic lymphocytopoietic factor. The assay method described should be useful in chemical characterization of this factor and in studies of its mode of action.

Miss Rowena Lichtenstein and Mrs. Florence Slater have provided valuable technical assistance

* This investigation was aided by grants from the Life Insurance Medical Research Fund (G64-67), the National Cancer Institute, National Institutes of Health, USPHS (CA-07470-02), the National Science Foundation (GB-2711), and the American Cancer Society (P-68F).

† Formerly Special Fellow of the U.S. Public Health Service.

‡ Postdoctoral trainee supported by training grant provided by the USPHS (5-Ti-GM-563-04).

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[11a] Injection of this extract (0.5 ml daily) into a group of 6 mice for 3 days resulted in a mean peripheral (tail vein) blood lymphocyte count of 7380/mm,^{3} while a control group of 7 mice, injected with Hank's solution, had a mean count of 4980 lymphocytes/mm.^{3}

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