IMMUNOGLOBULINS ON THE SURFACE OF LYMPHOCYTES

II. The Bone Marrow as the Main Source of Lymphocytes with Detectable Surface-Bound Immunoglobulin*

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Recent experimental studies employing immunofluorescence (1–3) and radioautography (1) have indicated the presence of immunoglobulins on the surface of small lymphocytes. The immunofluorescent techniques employed live cells, in suspension, that were reacted with anti-Ig sera conjugated to a fluorochrome dye. About 40–50% of spleen, 10% of lymph nodes, and 15% of peripheral blood lymphocytes of the mouse contained Ig on their surface membranes. The thymus, on the other hand, contained very few lymphocytes with detectable surface-bound Ig (3). Either most of the lymphocytes residing in the thymus did not have surface-bound Ig, or if they did, the Ig was present at a concentration or in a distribution that was not demonstrable by the immunofluorescent method. The function of the surface-bound Ig was not known although it was speculated that it could play a role in the recognition of antigen by lymphocytes.

The present study is concerned with the origin, that is whether derived from thymus or bone marrow, of the two types of lymphocytes, i.e., lymphocytes with or without detectable surface Ig. It appears that most of the lymphocytes that have detectable surface-bound Ig are derived from cells of the bone marrow while most lymphocytes without demonstrable Ig originate from the thymus. However, a small contribution of cells from each source cannot be

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excluded. Furthermore, quantitative measurement of light chain antigenic determinants on thymus cells indicates that if light chains are present on thymic cell surfaces at all, they are there in extremely small amounts; the highest value obtained in a series of experiments was 2.6% of that obtained for spleen cells.

Materials and Methods

Vital Immunofluorescent Technique.—Live lymphocytes were reacted with a fluorescein-labeled rabbit anti-mouse Ig sera (FITC anti-Ig)[1] in a small volume at room temperature, then washed and examined under the ultraviolet microscope. Complete details of the technical procedure as well as the results obtained in normal mice have been described (3). The FITC anti-Ig reacted with all classes of mouse Ig as well as with Ig kappa light chain.

An indirect or "sandwich" technique was employed to detect strain histocompatibility antigens. The antisera were obtained from CBA/St mice 7 days after the last of five weekly injections of 10^8 spleen cells from A/St mice. The technique consisted of first adding 4×10^6 spleen cells (in 20 μl of Eagle's medium containing 10% fetal calf serum) to 100 μl of CBA anit-A sera for 30 min at room temperature; the cells were washed three times, suspended in 20 μl of medium, and then mixed with 20 μl of FITC anti-Ig for 30 min. After three more washes the cell suspension was mounted on a glass slide and examined by fluorescence microscopy. As a control another portion of cells was incubated first with normal mouse serum (NMS) (instead of with the CBA anti-A serum) and then with FITC anti-Ig serum. By this procedure a difference in the number of fluorescent lymphocytes between the control and experimental cells would be obtained only if the histocompatibility antigens of the A strain were present on lymphocytes which would normally not show surface-bound Ig. Those lymphocytes which normally show detectable surface-bound Ig would obviously react positive in the control situation (NMS followed by FITC anti-Ig). Experiments designed to test the validity of this method using cells of CBA and A mice are given in Table I. Note that all thymus cells of A/St mice gave a positive reaction with CBA anti-A sera followed by FITC anti-Ig, while none showed a positive reaction in the control situation (NMS followed by FITC anti Ig); 95% of spleen cells from A/St reacted with CBA anti-A, while 48% reacted in the control procedure (NMS and FITC anti-Ig sera). Hence, all thymus cells of A mice have histocompatibility antigens and no detectable surface-bound Ig, while half of the splenic lymphocytes carried A antigens on their surface and no detectable Ig. The pattern of distribution of the histocompatibility antigens was patchy, as described by Cerottini and Brunner (4).

Conventional techniques were used to obtain cells from spleen, bone marrow (from femur and tibia), and lymph nodes. Cytological analysis was made on cells centrifuged onto glass slides (5) and stained by Giemsa. CBA, A, and (CBA $\times$ A)F_1 mice were obtained from Strong Research Foundation, La Jolla, Calif. BAF1 mice were obtained from Jackson Laboratories, Bar Harbor, Maine.

Quantitative Radioimmunoassay for Cell Surface Ig.—The technique employed was the same as previously described (3). In brief, viable lymphoid cells in suspension were mixed with a specific rabbit anti-mouse kappa chain antiserum for 16 hr in the cold. After the 16 hr incubation period, cells and antiserum were separated by centrifugation and a fraction of the antiserum incubated in the cold for a further 24 hr with 0.02 μg N of ^{125}I-labeled mouse IgG. The antigen–antibody complexes were precipiated by the addition of a high titered sheep anti-rabbit IgG antiserum and the amount of antigen bound to antibody was quantitated by

[1] *Abbreviations used in this paper:* FITC anti-Ig, fluorescein-labeled rabbit anti-mouse Ig sera; NMS, normal mouse serum; SRBC, sheep red blood cells; Tx, thymectomized.

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gamma scintillation counting. The amount of antiserum that was used would bind 50% of the ^{125}I-labeled mouse IgG. If no light chains were present on the cell surfaces, then the antibodies would be free to bind to the radioactive antigen (50%). However, this binding would decrease if the antibody had previously reacted with light chains on the cells. The quantity of light chains on the cells could be quantitated by comparing the degree of inhibition obtained with the cell suspension with that obtained by known concentrations of free light chains. As controls for nonspecific inhibition of the antigen–antibody reaction, rabbit spleen cells were included in each experiment in which the amount of lymphocyte-associated light chains was being quantitated.

TABLE I

Immunofluorescence of Lymphocytes Using CBA Anti-A Serum and FITC Anti-Ig

Cells		Technical procedure		Lymphocytes with positive reaction
Source	Strain	Step 1	Step 2	
				(%)
Spleen	CBA	NMS*	FITC anti-Ig*	47
Spleen	CBA	CBA anti-A*	FITC anti-Ig	45
Thymus	A	NMS	FITC anti-Ig	0
Thymus	A	CBS anti-A	FITC anti-Ig	97
Spleen	A	NMS	FITC anti-Ig	48
Spleen	A	CBA anti-A	FITC anti-Ig	95

This table shows a representative experiment in which live lymphocytes were first exposed to either NMS or serum from CBA mice immunized with A spleen cells; and then to FITC anti-mouse Ig antibody. Note that about half of spleen cells from an A mouse react with CBA anti-A but not with NMS. The lymphocytes that react with NMS are those that normally show surface-bound Ig.

* CBA anti-A = serum from CBA mice immune to A spleen cells; FITC anti-Ig = rabbit anti-mouse Ig conjugated to fluorescein isothiocyanate; NMS = normal mouse serum.

RESULTS

Thymectomized Mice.—We first investigated whether the presence or absence of a thymus would influence the number of lymphocytes with or without surface Ig. These experiments were made in adult mice, thymectomized, and then irradiated and transplanted with bone marrow cells, or in neonatally thymectomized mice.

CBA/St mice of 8 wk of age were thymectomized and after 2 wk were given 850 R of whole body irradiation (the mice were placed equidistant to two X-Ray sources of 200 kv that faced one another 160 cm apart; the dose of irradiation was 55 R/min). A few hours after irradiation, all mice were injected with 10^7 cells from the bone marrow of 6-wk old mice. A group also received 8.5×10^7 syngeneic thymocytes a month after irradiation and bone marrow transplantation. 8–12 wk after irradiation and bone marrow transplantation, the mice

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were sacrificed and their spleens and lymph nodes were examined by the vital immunofluorescent technique. Four to six mice from each group were examined.

The number of lymphocytes with surface Ig in spleen and lymph nodes of both groups was comparable. Spleens contained about 20–50 $\times$ 10^6 positive lymphocytes while lymph nodes (from axilla and inguinal nodes) contained 2–6 $\times$ 10^6 lymphocytes. However, the number of lymphocytes without detectable Ig was greatly decreased in the group of mice thymectomized but not

TABLE II

Lymphocytes with Surface Ig in Spleen and Lymph Nodes of Adult Thymectomized Mice

Organ examined	Treatment of mouse	Lymphocytes*	Cells with Ig‡	Lymphocytes with Ig	Absolute number of lymphocytes ($\times 10^{-6}$)	
					With Ig	Without Ig
		(%)	(%)	(%)		
Spleen	Tx	57.3	48.9	85.3	49.9	8.5
Lymph node		89.3	64.0	71.6	6.4	2.5
Spleen	Tx	55.0	56.5	100.0	33.9	—
Lymph node		88.9	70.0	78.7	4.9	1.3
Spleen	Tx and	77.3	43.2	55.9	31.5	24.7
Lymph node	thymocytes	98.7	35.0	35.5	3.5	6.4
Spleen	Tx and	70.0	37.4	53.4	25.8	22.2
Lymph node	thymocytes	98.0	31.9	32.6	1.9	4.0

This table shows the results of cytological analysis of four individual mice: the first two mice were thymectomized (Tx), irradiated; and reconstituted with bone marrow cells; the other two were treated in the same way but were also injected with thymocytes (Tx and thymocytes). The cytological analysis among spleen varied greatly from mouse to mouse; about 20–40% of cells in the spleen consisted of erythroid and myeloid elements. Normal untreated mice had about 45–52% of lymphocytes with surface Ig (50 $\times$ 10^6 cells). The inguinal and axillary lymph nodes of normal mice showed about 20% of lymphocytes with surface Ig, i.e., about 2 $\times$ 10^6 lymphocytes with detectable Ig.

* Determined in Giemsa-stained smear.

‡ Determined by immunofluorescence.

reconstituted with thymus cells (reduction of 75–100%). Because the thymectomized mice were depleted of the lymphocytes without surface Ig, they showed a relatively higher concentration of positive lymphocytes. Hence, about 85–100% of their spleen lymphocytes contained demonstrable surface Ig; in the thymectomized mice reconstituted with thymocytes about half of the splenic lymphocytes had surface Ig, a figure similar to normal mice. Lymph nodes of thymectomized mice had 72–79% of lymphocytes with surface-bound Ig; lymph nodes of thymectomized mice given thymocytes had about ¼–⅓ of lymphocytes with surface Ig. Results of the examination of two representative mice from each group are given in Table II.

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The presence or absence of lymphocytes with surface Ig was also investigated in neonatally thymectomized mice. Mice were thymectomized within 24 hr of birth. At 4–5 wk of age they were sacrificed and lymph nodes and spleen were examined by the vital immunofluorescent technique. Sham operated or normal littermates served as controls. The mediastinum was carefully examined at the time of sacrifice for the presence of residual thymic tissue. The results of this experiment were comparable to the previous ones. Neonatally thymectomized mice contained lymphocytes with surface-bound Ig but were greatly depleted of those lymphocytes not showing surface Ig. In fact, about 82–90% of the lymphocytes in the spleen and lymph nodes of thymectomized mice showed surface-bound Ig.

Adult Thymectomized CBA Mice Reconstituted with CBA or (CBA × A)F_1 Thymocytes.—The previous experiment indicated that an adult thymectomized and lethally irradiated mouse transplanted with syngeneic bone marrow cells would show a normal content of lymphocytes with detectable surface Ig. These mice if also transplanted with syngeneic thymocytes would show lymphocytes without detectable surface Ig in spleen and lymph nodes. In order to be able to more definitely identify thymic-derived cells as the source of the Ig-negative cell population, an experiment was performed similar to that described above except that the thymocytes came from an (CBA × A) F_1 hybrid. The thymic-derived cells in a spleen could then be identified immunohistochemically by means of antisera directed against the histocompatibility antigens of the A strain.

Adult CBA/St mice were thymectomized, irradiated, and transplanted with 10^7 bone marrow cells of CBA/St mice. 4 wk later mice received 10^8 thymocytes of either CBA/St or (CBA × A) F_1 hybrid mice. 6 wk later the mice were sacrificed and their spleens were obtained and examined for the presence of Ig with FITC anti-Ig, as well as for the possible presence of A antigens (a marker of the (CBA × A) F_1 thymocytes) by using CBA anti-A followed by FITC anti-Ig.

Thymocytes would normally be negative when stained with NMS followed by FITC anti-mouse Ig antiserum; however, when they were preincubated with anti-A antiserum and then with FITC anti-Ig, they became positive since their surface membranes contain histocompatibility antigens (Table I). In a mixed population of spleen lymphocytes, some with A antigens and some without but having surface Ig, if the number of positive cells when staining with CBA anti-A–FITC anti-Ig was higher than when staining with NMS–FITC anti-Ig this would be indicative of lymphocytes having A surface antigens and no Ig. On the other hand a comparable number of positive cells under both situations would indicate that lymphocytes having A antigens had acquired detectable surface Ig (provided that most lymphocytes were positive). As shown in Table III about 60% of lymphocytes had surface-bound Ig when stained directly with FITC anti-Ig. Note that the number of spleen cells that

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reacted with NMS–FITC anti-Ig and with CBA anti-A–FITC anti-Ig was comparable in the group in which the injected thymocytes were from a CBA donor. However, when the thymocytes came from a (CBA × A) F_1 donor, there was a significant difference between the number of spleen lymphocytes positive with NMS–FITC anti-Ig (63%) and with CBA anti-A–FITC anti-Ig (100%). Hence, apparently all of the Ig-negative population of cells in the spleen under these experimental conditions were of thymic origin.

Lethally Irradiated Mice Injected with Thymocytes plus Antigen.—The possibility was entertained that some thymic cells in peripheral lymphoid tissues

TABLE III

Immunofluorescence of Spleen Cells from Adult Thymectomized CBA Mice Reconstituted with Thymus Cells of Either CBA or (CBA × A)F_1

Source of thymocytes	Immunofluorescent procedure		Positive cells*	Lymphocytes in spleen‡	Positive lymphocytes
	Step 1	Step 2			
			(%)	(%)	(%)
CBA	NMS§	FITC anti-Ig§	55.4	80.9	68.4
	CBA anti-A§	FITC anti-Ig	50.0		61.8
(CBA × A)F_1	NMS	FITC anti-Ig	48.1	76.0	63.2
	CBA anti-A	FITC anti-Ig	76.7		100.0

All mice were thymectomized, irradiated, transplanted with bone marrow, and then given either CBA or (CBA × A)F_1 thymocytes. This table shows a representative experiment in which spleen cells of two mice were pooled and analyzed cytologically and immunohistochemically. The immunofluorescent procedure is the same one described in Table I.

* Percentage of all spleen cells that have positive surface fluorescence.

‡ Percentage of lymphocytes among spleen cells as determined by Giemsa examination of smears.

§ CBA anti-A = serum from CBA mice immune to A spleen cells; FITC anti-Ig = rabbit anti-mouse Ig conjugated to fluorescein isothiocyanate; NMS = normal mouse serum.

would show detectable surface Ig only after reaction with antigen. To test for this possibility experiments were made in which thymocytes plus antigens were injected into lethally irradiated mice; several days later the cells from the spleen were harvested and examined by immunofluorescence. This experimental procedure is known to produce a population of thymus-derived cells in the spleen that react specifically with antigen, do not form antibodies, but "cooperate" with bone marrow-derived cells for antibody production (see review in reference 6).

Two series of experiments were made. First, 8-wk old BAF_1 mice were irradiated with 1000 R. Each of eight mice was then injected intravenously with 1.2×10^7 thymocytes; four were also injected with 2×10^8 sheep red blood cells (SRBC). A third group of four mice did not receive thymocytes nor

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SRBC. 8 days after irradiation the mice were sacrificed, the spleen cells were harvested and examined by vital immunofluorescence for surface Ig. Table IV shows these data. A low number of positive cells were seen in all groups, i.e., 1–6%. When calculated on the basis of total positive cells per spleen, however, all groups had approximately the same number of lymphocytes with detectable surface Ig.

A second series of experiments were made with CBA/St mice and using keyhole limpet hemocyanin as the antigen. Hemocyanin is a markedly thymus-dependent antigen (7). CBA/St mice were irradiated with 850 R and then injected intravenously with 10^8 thymocytes and 1 mg of hemocyanin. Control mice received thymocytes but no antigen. 6 days later the mice were sacrificed, the spleens were obtained, and their cells were harvested and studied by immunofluorescence. The results, not shown in Table IV, were comparable to

TABLE IV

Lymphocytes with Surface Ig in Spleens of Mice Irradiated and Injected with Thymocytes

Treatment of mice			Lymphocytes with Ig	No. of lymphocytes in spleen ($\times 10^{-6}$)	Lymphocytes in spleen with Ig ($\times 10^{-4}$)
Irradiation	Thymocytes	Sheep red blood cells			
R			(%)		
1000	1.2×10^7	2×10^8	1	4.1	4.0
1000	1.2×10^7	None	1	5.0	5.0
1000	None	None	6	1.5	9.0

The spleens of four mice from each group were harvested 8 days after treatment. The cells were pooled and examined by Giemsa and by immunofluorescence.

the previous experiment. Only about 1–4% of cells in the spleen had detectable surface-bound Ig (four mice from each group were examined) regardless of whether or not hemocyanin had been injected. Hence, spleen cells from lethally irradiated mice which had received thymocytes and antigen did not show an increase in number of lymphocytes with detectable surface Ig.

Quantitation of Light Chains on Surfaces of Thymocytes.—Since some experiments have been reported which suggest thymus cells have light chain determinants associated with them (8), a quantitative immunoassay (described previously in reference 3) was used to detect and measure the light chains that might be present on the surface of thymocytes. Table V presents these data. As controls for nonspecific inhibition of the radioactive antigen–antibody system, rabbit spleen cells were also tested. The fourth and fifth rows represent the data in terms of nanograms of light chains per 10^6 cells, after subtraction of the values obtained with the rabbit spleen cell controls. In two experiments there was no difference between the inhibition caused by thymocytes and the rabbit spleen cell controls, whereas in the other three experiments the thy-

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mocytes showed slightly more inhibition than the control cells. The values for thymocytes ranged from 0.0 to 0.016 ng N/10^6 cells compared with values of 0.195–1.015 for spleen cells. In terms of relative amounts, therefore, thymocytes contained from 0.0 to 2.6% of the amount of Ig contained on spleen cells. The degree of inhibition exhibited by thymocytes was of such low order of magnitude that it is not possible to conclude whether it was significant at all, or whether it could be due to a minute amount of blood cells and/or serum in the preparations.

DISCUSSION

The present results indicate that the majority of lymphocytes with Ig on their membranes as demonstrated by the immunofluorescent method derive from the bone marrow, while most of those lymphocytes having no detectable

TABLE V

Quantitation of Light Chains on Mouse Thymocytes

Cells tested	Light chains (ng N/10^6 cells)				
	Experiment Number				
	1	2	3	4	5
Mouse spleen cells	0.205	0.202	1.021	0.286	0.610
Mouse thymus cells	0.012	0.006	0.017	0.006	0.016
Rabbit spleen cells	0.010	0.006	0.006	0.008	0.000
Mouse spleen cells (corrected	0.195	0.196	1.015	0.278	0.610
Mouse thymus cells (corrected)	0.002	0.000	0.011	0.000	0.016
Thymus/spleen × 100	1.0	0.0	1.1	0.0	2.6

Ig are thymic derived. It was clear that thymectomized mice were greatly depleted of lymphocytes without detectable Ig, while having a normal number of lymphocytes with surface Ig. Also, the transplantation of thymic lymphocytes into thymectomized mice did not result in an increase in the number of lymphocytes with surface Ig. Clearly, in one such experiment, many splenic lymphocytes had the histocompatibility marker of transplanted thymocytes without showing surface Ig. Hence, many thymus cells after residing in peripheral lymphoid tissue for many days did not appear to acquire surface Ig at a detectable level. Similar conclusions on the origin of lymphocytes with or without detectable Ig has been reached by Raff using the theta antigen as a marker of thymus-derived cells (9).

Since it is apparent that the thymus-deprived mice are not lacking lymphocytes with surface Ig, it follows, therefore, that these mice have at least the potential to manifest the function of these lymphocytes. It is known that thymectomized mice respond poorly to many antigens although they do not lack the cells that make antibody (10). It is likely that the lymphocytes with

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surface Ig represent the same population of potential (or active) antibody-forming cells. Along these lines, it should be remembered that thymus-deficient mice have normal levels of Ig (11) and of natural antibodies (12). This finding of no Ig on thymocytes should be analyzed with respect to their apparent capacity to recognize and discriminate antigens. The following explanations, some of which are not mutually exclusive, are compatible with the present findings: (*a*) The thymus cells have Ig but at a concentration or in a distribution below the level of detection of the immunofluorescence technique or radioactive immunoassay. If this is the case the amount of Ig is on the order of 3% or less of that present on spleen cells (or in other terms a few hundred Ig molecules per cell). (Our quantitative immunoassay for light chains was equivocal in that, although it placed an upper limit on the amount of Ig on the surface of thymocytes to a few hundred molecules per cell, it did not rule out the presence of these few hundred molecules, or if Ig was limited to a relatively small percentage of thymocytes considerably more Ig could be present on the positive cell population). (*b*) The thymus may have a large population of cells without surface Ig and a small population of cells with surface-bound Ig. This minor population, which may be the one responsible for antigenic recognition, is not demonstrable under our experimental approaches because it is obscured by the large number of Ig-positive cells derived from the marrow. (*c*) Or thymus cells recognize antigen by receptors that are unlike known immunoglobulins in that they do not have recognizable light chain determinants. Whatever the correct explanation is, it is nevertheless apparent that there are distinct differences among thymus- or bone marrow-derived cells with respect to their content of surface bound Ig.

If the results obtained in the present study are taken at face value, i.e. negligible amounts of light chains are associated with thymocytes, they are at variance with data that suggest that anti-light chain antisera abrogate the ability of thymus cells to participate in a graft-vs.-host reaction (8) and inhibit thymic-dependent reactions of peripheral blood lymphocytes such as the mixed lymphocyte reaction (13). In contrast, however, other workers have been unable to block the responsiveness of thymus cells with anti-light chain reagents (14)[2]. The apparent nonuniformity of light chain antisera to be active in these assays suggests that perhaps when occasional antisera are observed which are active, they act because they also contain antibodies to some antigen other than light chains which is present on lymphocyte surfaces. Continued experience with such active reagents will be needed before an answer to this problem will be obtained.

SUMMARY

Immunofluorescent studies using live cells from antibody-forming organs and anti-immunoglobulin antibodies demonstrate two populations of small

[2] Cerrottini, J. C., and K. T. Brunner. Personal communication.

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lymphocytes which are differentiated by the presence or absence of Ig on their surface membranes. Most of the lymphocytes with detectable surface Ig appear to derive from cells of the bone marrow, while most of the Ig-negative lymphocytes derive from the thymus. Thus, adult mice thymectomized, lethally irradiated, and transplanted with bone marrow cells showed a normal number of lymphocytes with surface Ig but were depleted of the Ig-negative lymphocytes. Injection of thymocytes into these mice did not result in an increase in the number of lymphocytes with surface Ig in spleen and lymph nodes. Most of the injected thymocytes could be identified by means of histocompatibility markers. Also, the spleen and lymph nodes of neonatally thymectomized mice contained lymphocytes with surface Ig but were depleted of the Ig-negative lymphocytes.

Attempts were made to identify light chains on thymocytes by a sensitive radioimmunoassay. In some experiments no light chains were detected and in others a small amount, i.e. no more than 2.6% of the amount present on spleen lymphocytes, could be detected. Whether these low figures are significant or represent a small amount of serum contamination is not clear as yet.

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