STUDIES ON CHARACTERIZATION OF THE LYMPHOID TARGET CELL FOR ACTIVITY OF A THYMUS HUMORAL FACTOR*

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Participation of thymus humoral factors (THF)[1] in the conferment of immune reactivity has been unequivocally demonstrated in the past few years. The original assumptions of a humoral factor activity stemmed from studies of partial recovery of cell-mediated and humoral-immune reactions in thymectomized mice implanted with thymus tissue in cell impermeable chambers (1–4). Indeed, it was subsequently demonstrated that administration of extracts from thymus tissue of different animal origin to neonatally thymectomized mice prevented wasting disease (5), and partially restored the ability to produce a primary immune response to sheep red blood cells (SRBC) (6, 7), as well as to reject skin and tumor allogeneic grafts (8). Furthermore, spleen cells from neonatally thymectomized mice gained the capacity to elicit in vitro a graft-vs.-host (GvH) response, when incubated in dialyzed THF preparations (9). Attempts to characterize the target cell for the activity of THF in this system showed that bone marrow cells treated with THF and transferred through an intermediate recipient acquired immunological competence, as measured in the same GvH in vitro system (10). These observations conform with the conclusions derived from in vitro studies on interactions of thymus with bone marrow and spleen tissue (11). However, the exact identity of the target cell for THF activity within the bone marrow cell population is still obscure, since the bone marrow of normal animals contains precursors of B lymphocytes, as well as some T lymphocytes (12, 13). The question therefore arises, which cell type is affected by THF. Does THF contribute to the maturation of B or T, or both lymphoid cell types? Furthermore, in view of the concept that lymphocytes of the thymus in the adult develop from cells of bone marrow origin (14), we attempted to test whether differentiation to active T cells within the thymus depends entirely on the effect of THF. Our preliminary observations in this study have been reported (15).

Materials and Methods

Mice.—(BALB/c $\times$ C57BL/6)F$_1$, at the age of 8–10 wk, were used throughout these experiments. Thymectomy was performed under Nembutal anesthesia and the thymus was removed by suction. At the end of the experiment any animal found to contain a thymic remnant was discarded.

X Irradiation.—Mice were exposed to total body irradiation 7 days after thymectomy. The animals were given a single dose of 750 R from a ^{60}Co source (gamma beam 150 A, Atomic Energy of Canada Ltd., Ottawa, Ontario, Canada) 60 R/min, focal skin distance, 34 inches.

Preparation of Cells.—Thymus and bone marrow cell suspensions were prepared from syngeneic (BALB/c $\times$ C57BL/6)F$_1$ donor mice as previously described (16), and were injected intravenously. In most of the experiments thymus cells were administered immediately after irradiation, whereas the bone marrow cells were given 7 days later.

Immunization and Assay.—0.1 ml of a 10% suspension of SRBC was injected intraperitoneally twice, 24 h and 7 days after irradiation. The number of hemolytic plaque-forming cells (PFC) was determined by Jerne's technique (17), 5–6 days after the second injection of antigen. The schedule used in these experiments is described in Fig. 1.

Treatment with Thymus Humoral Factor (THF).—Extracts from calf thymus were prepared and dialyzed as previously described (18). In addition, certain fractions obtained by methanol chloroform extraction were also used. The preparations employed contained 1 mg protein per milliliter dialyzed THF. Every sample of THF was tested for reactivation of the ability of spleen cells from neonatally thymectomized mice to elicit an in vitro GvH response (9). In vivo
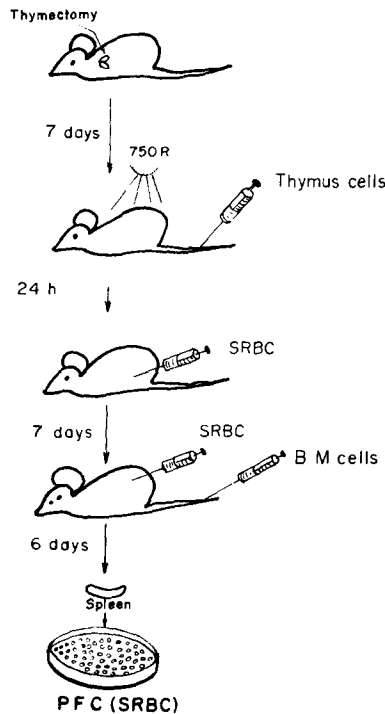

Fig. 1. Schedule of experimental procedures.

treatment: mice were injected intraperitoneally with 0.5–1.0 ml of THF. In vitro treatment: cells were incubated for 1 h at 37°C with 2% THF, in Eagle's medium supplemented with agamma globulin horse serum, washed and resuspended in Eagle's medium free of THF.

Statistical Evaluation.—The results obtained were submitted to Student's t test for evaluation of significance.

RESULTS

Previous experiments performed in this laboratory have shown that treatment with THF of spleen cells from neonatally thymectomized mice restored their competence to induce a cell-mediated immune response as measured in a GvH reaction in vitro (9). It has been suggested that THF acts on precursors of T cells, since the T cells were reported to participate in the GvH response (19). However, during the activation process by THF, no increase in the number of θ-bearing cells was observed in the treated cell population (20). Two alternative hypotheses could explain these results: (a) THF permits bone marrow cells to differentiate to reactive T cells which do not possess the θ-antigen, or (b) THF induces functional maturation of cells which already carry the θ-antigen. According to the first hypothesis we assumed that if bone marrow cells can develop into T cells after treatment with THF, such treated cells should be able to interact with B cells in response to antigenic stimulation, in a system which requires cooperation of T and B cells (21, 22). To test this possibility, thymectomized mice were lethally irradiated, and injected with 30×10^6 bone marrow cells, 0.5 ml THF, and 0.1 ml 10% SRBC. Additional treatment with the same amount of bone marrow cells and SRBC without THF was repeated a week later (group I, Fig. 2). Controls included one group treated as above but without THF (group II), a group which was not injected with SRBC (group III), a group which received two injections of bone marrow cells only (group IV), and a last one that served as positive controls, in which thymic cells were given instead of the first injection of bone marrow cells. Experimental and control animals were sacrificed 5 days after the second injection of antigen, and their spleens were assayed for the number of PFC. As shown in Fig. 2, spleens of mice injected with bone marrow and THF (group I) did not manifest any increase in the number of PFC, as compared to animals that were not injected with THF (group II). A significant response was obtained only in animals injected first with thymic cells instead of bone marrow cells (group V). It was, therefore, concluded that under the experimental conditions employed, bone marrow cells subjected to THF treatment in the absence of intact thymus tissue do not function as T cells.

These results could be attributed to the possibility that the number of target cells for THF effect in the bone marrow is too low to be detectable by T helper function in this system. The same experiment was therefore performed with higher numbers of bone marrow cells given at the first injection. Thymectomized, irradiated mice were thus inoculated with 10^8 bone marrow cells, and injections of THF and SRBC followed as in the previous experiment (group I,

Table I). A control group was injected with the same number of bone marrow cells without THF (group II), and another control group received 5×10^6 thymus cells and SRBC (group III). All animals were given 30×10^6 bone marrow cells and SRBC 1 wk later. The results obtained clearly indicate that

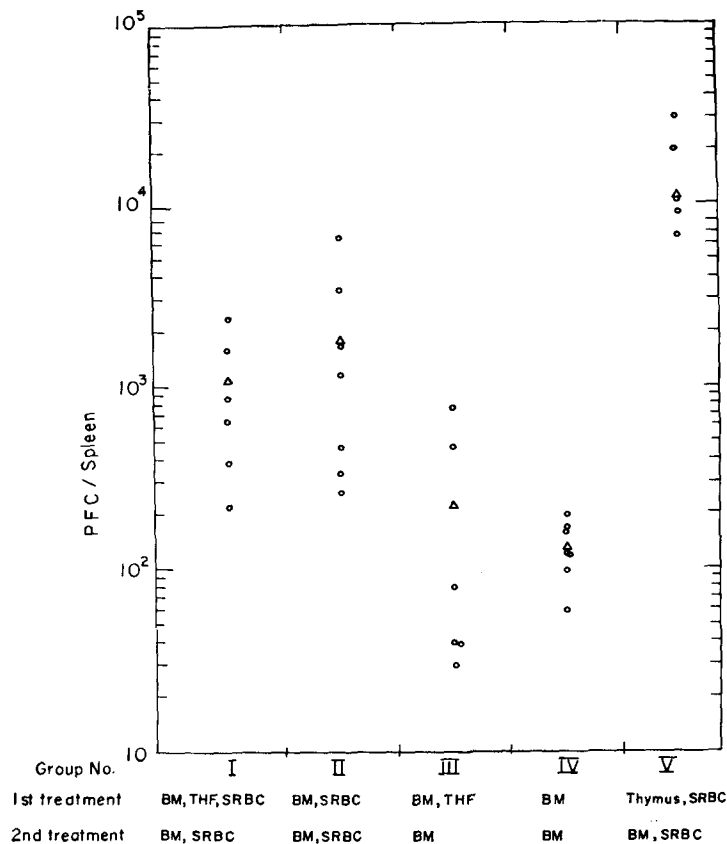| Group No.	I	II	III	IV	V
1st treatment	BM, THF, SRBC	BM, SRBC	BM, THF	BM	Thymus, SRBC
2nd treatment	BM, SRBC	BM, SRBC	BM	BM	BM, SRBC

FIG. 2. Antibody response to SRBC measured in the spleen of thymectomized irradiated mice injected with BM cells and THF. $\bigcirc$ = number of PFC in individual spleens. $\triangle$ = mean.

TABLE I

Number of PFC in the Spleen of Thymectomized Irradiated Mice Injected with High Numbers of Bone Marrow Cells and THF

Group	Treatment*	No. of PFC/spleen	No. of animals
I	10^8 BM, 0.5 ml THF	380 (20–1,040)	9
II	10^8 BM	295 (80–1,000)	10
III	5×10^6 thymus	4,565 (1,900–6,000)	3

* All animals were injected with SRBC and 1 wk later inoculated with 30×10^6 bone marrow cells and SRBC again.

even when higher numbers of bone marrow cells were used, no effect of THF on these cells could be observed.

Consequently, in the next series of experiments we decided to test the alternative hypothesis, namely, that THF induces differentiation of thymocytes to T cells. The system employed was similar to that used in the previous experiments. Thymectomized, irradiated mice were inoculated with 10^8 thymus cells and in addition, received 0.5 ml THF and 0.1 ml 10% SRBC. The mice were subsequently injected with 30×10^6 bone marrow cells and once more stimulated with SRBC (group I, Fig. 3). Controls included one group as above, but
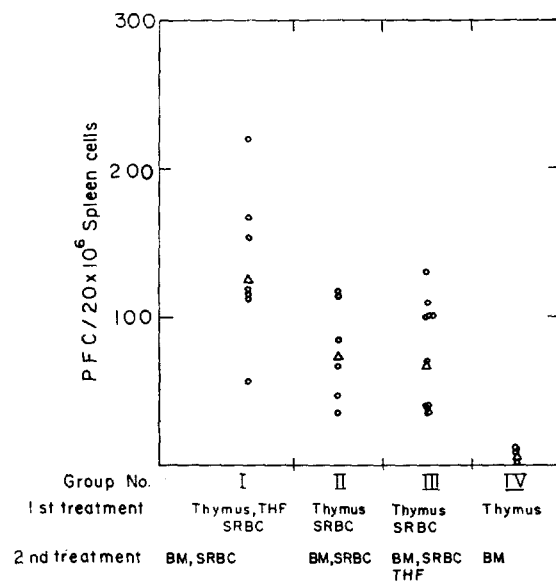
Fig. 3. Antibody response to SRBC measured in the spleen of thymectomized irradiated mice injected with thymus cells and THF. O = number of PFC in individual spleens. △ = mean.

without THF (group II), another control group consisted of animals which were first given 10^8 thymus cells only, whereas THF was given together with the bone marrow cells in the second injection (group III), and a last control group (IV) in which the animals received thymus cells and bone marrow cells without any injection of SRBC.

As shown in Fig. 3, simultaneous administration of THF and thymus cells (group I) increased significantly the number of PFC, as compared to control group II, suggesting that THF increases the helper effect of the thymus cell population. This effect of THF on cell maturation appears to be specific for thymic cells, since THF given simultaneously with bone marrow cells (group III) did not increase the number of PFC. Therefore, the possibility that THF

affected bone marrow cells and thus increased the population of mature B cells does not seem plausible under the present experimental conditions.

When these experiments were repeated with a constant low dose of thymocytes followed by inoculation with bone marrow cells incubated with THF, no increase in the number of PFC was observed (Table II). Thus, we could not detect any effect of THF on bone marrow cells.

In order to find out whether THF acts directly on the target thymus cells through direct contact, or whether the effect is indirect, we tested whether exposure of the thymus cells to THF in vitro would also result in an enhanced immunological activity. Thymus cells at a concentration of 30 $\times$ 10^6 cells per milliliter were incubated in Eagle's medium supplemented with horse serum and with THF for 1 h at 37°C with continuous shaking. The cells were then washed and inoculated at a dose of 10^8 cells into thymectomized, irradiated recipients challenged with SRBC. Subsequently, the animals received bone

TABLE II

Number of PFC in the Spleen of Thymectomized Irradiated Mice Injected with Thymus Cells and Subsequently with Bone Marrow Cells Incubated with THF

Group	Treatment of BM cells*	No. of PFC/spleen	No. of animals
I	Incubation in THF	964 (280–2,010)	7
II	Incubation in spleen extract	1,185 (450–2,100)	7
III	Incubation in medium	685 (650–720)	4
IV	Cells kept at 4°C	1,143 (870–2,250)	5

* All animals received 5 $\times$ 10^6 thymus cells and antigen a week before inoculation of treated 30 $\times$ 10^6 bone marrow cells.

marrow cells and an additional injection of SRBC. (Fig. 4, group I). In one control group, thymus cells were incubated under similar conditions, but in the absence of THF (II). In another control group thymus cells were incubated with THF at 4°C (III), whereas in group IV thymus cells were kept at 4°C without THF. In the last group (V) untreated thymocytes were injected with bone marrow cells without SRBC. The results obtained indicate that incubation of thymus cells with THF at 37°C caused a significant increase in the number of PFC, as compared to thymus cells incubated under the same conditions but without THF. This effect seems to be temperature-dependent, since incubation with THF in the cold did not exert a similar effect. The possibility that thymic cells were damaged when kept in the cold, and were therefore inactive, was ruled out, since group III manifested the same activity as did the control group of thymus cells incubated at 4°C without THF (group IV).

From the data accumulated in the above experiments it appears that there is a significant increase in the number of PFC when thymus cells are treated with THF either in vivo or in vitro. However, in the system used we measured the

number of antibody-producing cells, which represents the activity of the bone marrow-derived cell population. Consequently, it was important to evaluate quantitatively the increment in helper T cell activity.

In order to clarify the relationship between an increase in the number of active T cells and the resulting number of PFC, an experiment was designed using progressive doses of thymus cells (10^6, 5 $\times$ 10^6, 10^7, 5 $\times$ 10^7, and 10^8). The cells were inoculated into thymectomized irradiated mice, challenged with SRBC, and subsequently injected with a constant number of 30 $\times$ 10^6 bone
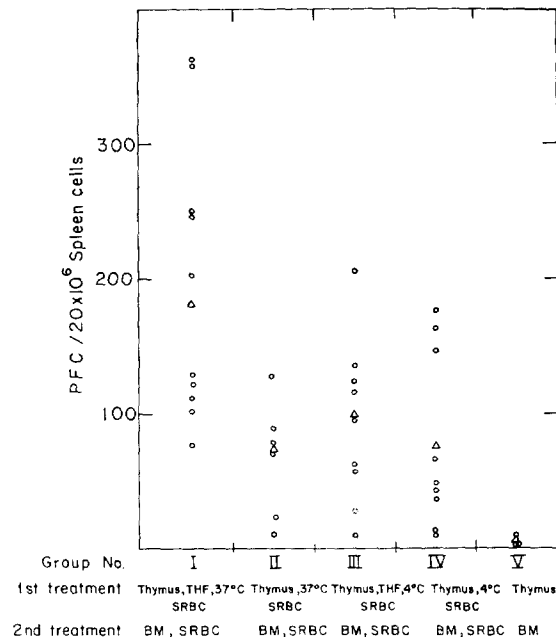

FIG. 4. Antibody response to SRBC measured in the spleen of thymectomized irradiated mice injected with thymus cells previously incubated with THF in vitro. $\bigcirc$ = number of PFC in individual spleens. $\triangle$ = mean.

marrow cells. The results of this experiment are summarized in Fig. 5. As can be seen in the figure, a 10-fold increase in the number of thymus cells injected resulted in an 80% increase in the number of PFC, and at the higher doses of cells the curve reached a plateau.

In view of these results, we decided to investigate the effect of THF on low concentrations of thymus cells. Thymectomized irradiated mice were inoculated with 5 $\times$ 10^6 thymus cells and simultaneously injected with 0.5 ml of THF. On the next day the animals were challenged with SRBC and subsequently injected with 30 $\times$ 10^6 bone marrow cells (group I). Control groups included one group similar to the experimental one, but without THF treatment (group II), and

another consisting of animals injected with thymus and bone marrow cells only (group III). As seen in Fig. 6, animals receiving THF together with the thymic cells showed a striking increase in the number of PFC, as compared to that observed in the control groups. In the next experiment thymus cells were
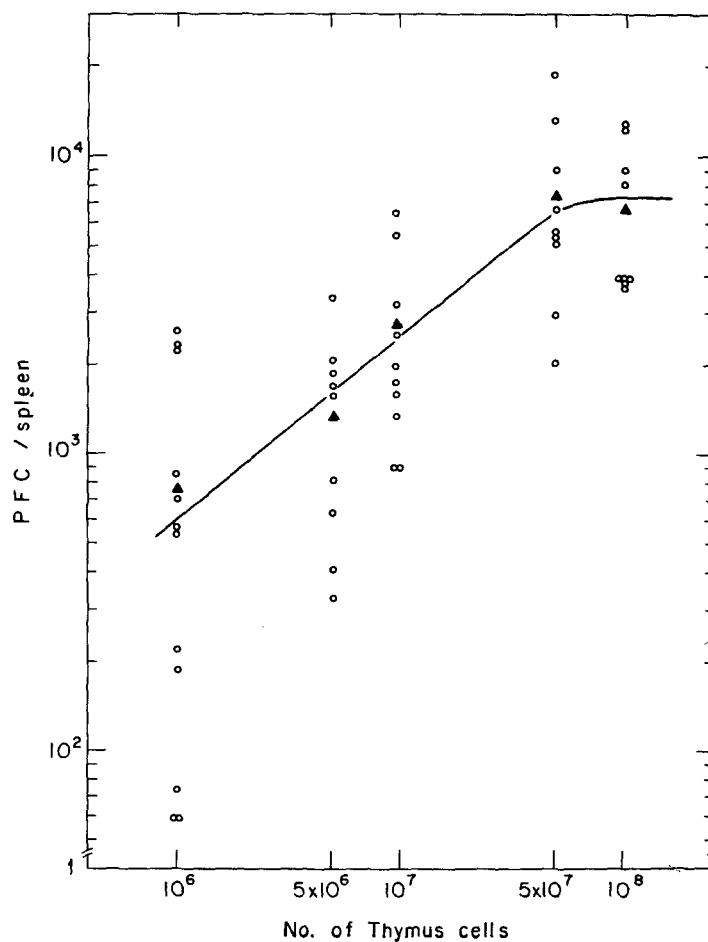

FIG. 5. Antibody response to SRBC measured in the spleen of thymectomized irradiated mice injected with progressive numbers of thymus cells and constant numbers of BM cells. O = number of PFC in individual spleens. ▲ = mean.

incubated at a concentration of 30×10^6 cells/milliliter at 37°C in the presence of THF. The cells were washed and injected in doses of 15×10^6 cells per animal group (I, Table III). In one control group cells were incubated in the presence of an inactive fraction of THF, at the same protein concentration (group II). Another control group consisted of thymus cells incubated in
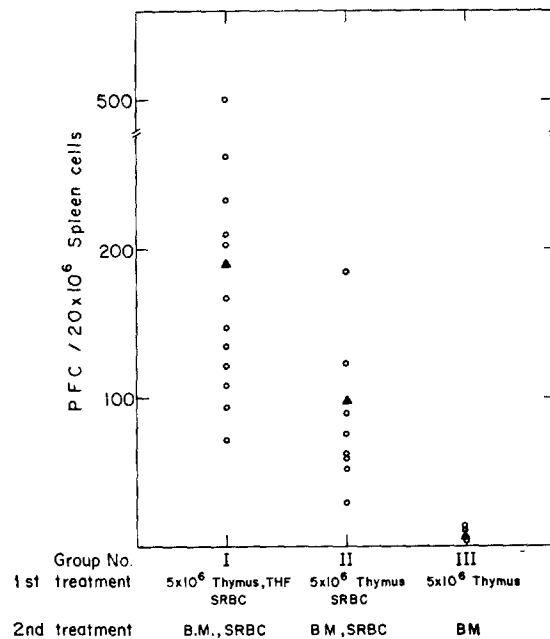
FIG. 6. Antibody response to SRBC measured in the spleen of thymectomized irradiated mice injected with low doses of thymus cells and treated with THF. ○ = number of PFC in individual spleen. ▲ = mean.

TABLE III

Number of PFC in the Spleen of Thymectomized Irradiated Mice Injected with Low Doses of Thymus Cells Incubated in THF

Group	Incubation of thymus cells*	Exp. 1 No. of PFC/spleen	Exp. 1 No. of animals	Exp. 2 No. of PFC/spleen	Exp. 2 No. of animals
I	THF active fraction	7,590 (6,780–12,060)	6	3,301 (350–9,500)	9
II	THF inactive fraction	1,875 (495–3,390)	6	610 (150–1,850)	7
III	Medium	2,958 (2,400–4,260)	5	1,483 (250–2,500)	8
IV	Cells kept in the cold	2,384 (1,530–3,510)	6	762 (300–1,500)	4

* All animals received 15×10^6 treated thymus cells and were challenged with SRBC, and 1 wk later the animals were injected with 30×10^6 bone marrow cells and SRBC again $(P \ll 0.01)$.

medium only (group III). In the last control, the thymus cell suspension was kept at 4°C (group IV). All animals were challenged with SRBC on the day after thymus cell inoculation, and 1 wk later the animals were injected with 30×10^6 bone marrow and again with antigen. The results of the two experi-

ments presented above indicate that incubation of thymus cells in the presence of the active fraction of THF caused a striking increase in the number of PFC. Thus, a significant increase in the number of PFC, which was manifested after in vivo as well as in vitro treatment of low numbers of thymic cells with THF, demonstrates unequivocally that THF enhances the activity of T cells even when the cells were applied in limited numbers.

DISCUSSION

The results accumulated in the present experiments clearly indicate that THF activates thymus cells to acquire a higher T helper cell activity, as measured by the response to SRBC, since such treatment caused a significant increase in the number of PFC in the experimental system used. On the other hand, bone marrow cells did not manifest any T cell activity after THF treatment under similar conditions. Nor did THF affect B cell activity as shown from the response of animals reconstituted with normal thymus cells, and THF-treated bone marrow cells which was not higher than that of control animals receiving the untreated bone marrow.

While in these experiments no effect of THF on bone marrow cells was demonstrated, it has been previously demonstrated that T cell function was acquired by bone marrow cells after THF treatment when tested in the in vitro GvH assay (9). Whether these results indicate two different target cell populations for the activity of THF is open to discussion. It is possible that in the GvH response the target cell is not a precursor of a T cell, whereas in the humoral response, THF contributes to further maturation of thymus cell populations only. Since the bone marrow cell population used in the GvH experiments of Small and Trainin (10) were not previously treated with anti-θ antiserum, it is possible that THF activated a minor population of T cells which is known to exist in the bone marrow of mice (12, 13, 23), and these may actually be the active cells in eliciting the GvH response. Furthermore, Burleson and Levey (24) reported that when bone marrow cells of mice were fractionated on bovine serum albumin gradient, an active cell population was obtained, capable of inducing a GvH response in vivo in newborn mice. Similarly, Yoshida and Osmond (25), working with Lewis rats (Lewis Corp., Woodbury, Conn.), demonstrated that lymphocyte-rich fractions separated from the bone marrow by sucrose gradient, manifested a high GvH activity, as measured by popliteal lymph node enlargement. These experiments indicate that a certain cell population within the bone marrow is able to cause a GvH response, but the detection of this capacity is possible only after its concentration by a variety of methods.

With regard to the antibody response to SRBC, it is generally accepted that antibodies are produced in thymectomized irradiated mice if bone marrow cells are administered together with thymus cells (1–4). However, Playfair obtained some degree of humoral response to SRBC (26) when bone marrow cells only

were injected at very high doses. It is possible that the T cells for cell-mediated reactivity, and the T helper cells for humoral response, represent different types of cell populations, as suggested by Segal et al. (27). Another possibility is that both in the cellular and the humoral responses the same type of T cell is involved, yet much higher doses of bone marrow cells are required for a detectable T cell response in antibody production. Accordingly, while bone marrow cells acquire a high level of T cell function after THF treatment, as expressed in the GvH response (9), this may still be insufficient to perform a T helper function for the humoral response. The fact that THF can induce T cell activity without any effect on other manifestations of this population was demonstrated by Lonai et al. (20). Treatment of different lymphoid cell populations with THF did not cause any increase in the number of θ-positive cells, although they acquired the capacity to elicit a GvH response. This suggests that, in the GvH reaction system, THF endows a sector of the bone marrow cell population with T cell properties, without acquiring a T cell marker such as the θ-antigen, and with no effect on the function in the humoral response. Acquisition of these activities by the bone marrow population may require an additional phase of differentiation.

Finally, the fact that incubation of thymus cells with THF at 37°C for 1 h only was sufficient to increase their T helper function, demonstrates that in this system, as well as in the GvH response (10), THF triggers a critical step, involving some metabolic process, since treatment at 4°C did not lead to any enhancement of the response.

SUMMARY

The immune response to SRBC was measured in the spleens of adult thymectomized, total body irradiated mice injected with various combinations of thymus and bone marrow cells together with thymic humoral factor (THF). It was found that the number of plaque-forming cells was significantly increased when THF was given in vivo immediately after thymus cell administration or when thymus cells were incubated in THF before injection. On the other hand, bone marrow cells equally treated did not manifest any T cell activity, since THF-treated bone marrow cells were not able to substitute thymus cells in the system used. The results accumulated in the present experiments indicate, therefore, that the target cells for THF activity are thymus cells which acquire a higher T helper cell capacity after THF treatment.

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