CELL TO CELL INTERACTION IN THE IMMUNE RESPONSE

II. The Source of Hemolysin-Forming Cells in Irradiated Mice Given Bone Marrow and Thymus or Thoracic Duct Lymphocytes*, ‡

By G. F. MITCHELL and J. F. A. P. MILLER, M.B.

(*From The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia*)

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An interaction between thymus or thoracic duct cells and antibody-forming cell precursors has been implicated in the response of mice to sheep erythrocytes (1). The possibility was raised that the thymus contributes cells to the circulating pool of lymphocytes which recognize antigen (ARC) and which influence the differentiation of antibody-forming cell precursors (AFCP) to hemolysin-forming cells. The experimental system employing reconstituted neonatally thymectomized mice failed to detect the existence of AFCP in inoculated lymphoid cell suspensions.

Previous work from this laboratory (2) had demonstrated that thoracic duct and spleen cells were capable of affecting the appearance of hemolytic foci in the spleens of heavily irradiated recipients injected with sheep erythrocytes. If such foci represent clusters of antibody-forming cells, then either the inoculum must contain cells potentially capable of producing hemolysins, or the host must provide AFCP which are extremely radio-resistant.

In this paper, we present the results of experiments designed to determine whether both ARC and AFCP are present in populations of cells from thymus, thoracic duct lymph, or bone marrow.

Materials and Methods

Animals.—Mice of the highly inbred strains CBA and C57BL, and F_1 hybrids from crosses between these strains were used. The origin and maintenance of these mice have been described in the previous paper (1).

Cell suspensions were prepared as before (1).

Operative Procedures.—Thymectomy or sham operation was performed in young adult mice, 4–6 wk old, as previously described (3). Checks for the presence of thymus remnants

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were made at autopsy and mice with such remnants were discarded from the experiments. Thoracic duct cannulation was performed as described in the previous paper (1).

Irradiation.—Intact or adult thymectomized mice were exposed to total-body irradiation in a Perspex box. In the case of thymectomized mice, the irradiation was performed 1–3 wk after thymectomy. The dose given was 800 rads to mid-point with maximum back scatter conditions and the machine operated under conditions given in the previous paper (1). The focal skin distance was 50 cm and the absorbed dose rate was 170 rads per minute. When thymectomized irradiated mice were to be protected with bone marrow, they received an intravenous injection of 2–5 million cells in 0.1–0.2 ml Dulbecco's phosphate-buffered saline 1–3 hr postirradiation. All irradiated mice were given oxytetracycline[1] (100 mg per liter) or penicillin[2] (600,000 units per liter) in the drinking water.

Assays for Hemolysin Plaque-Forming Cells and Hemolytic Foci.—Hemolysin plaque-forming cells were assayed according to the technique of Jerne et al. (4). The hemolytic focus assay used in the present experiments was a modification of the techniques described by Kennedy et al. (5) and Playfair et al. (6). The spleens were not frozen but serially sectioned into 250-μ segments by means of a tissue chopper[3] and sequentially transferred with forceps to an agar plate. These plates were then heated to 37°C and double the quantity of the top, soft agar layer as used in the Jerne assay, was poured over the segments. This layer contained 3.8 ml of 1.4% agar in double strength Eagle's medium, 2 mg dextran, and 0.2 ml of 20% sheep erythrocytes. After incubation for 1–2 hr, 1:10 guinea pig serum was added and the plates incubated for a further hour. The serum was then removed and the plates cooled to 4°C. The red cells were stained with benzidine[4] for 1–2 min to increase the contrast between the areas of hemolysis which had developed around the segments and the intact red cells. Immediately after staining, the hemolytic areas were counted with the aid of a dissecting microscope.

Preparation of Anti-H2 Sera and Incubation of Plaque-Forming Cells.—The methods for preparing specific anti-H2 sera and for incubating plaque-forming cells were given in the preceding paper (1).

Statistical Analysis.—The standard errors of the means were calculated and P values determined by Student's t test.

RESULTS

Effect of Syngeneic Thymus, Thoracic Duct, or Bone Marrow Cells in Irradiated Hosts.—10 million CBA thoracic duct cells, thymus cells, or bone marrow cells were injected together with 10^8 SRBC[5] into groups of syngeneic recipients that had been exposed to 800 rads total-body irradiation. The number of PFC per spleen was determined 7 days later. As seen in Table I, 10 million thoracic duct cells produced on the average 1270 PFC whereas 10 million thy-

[1] Terramycin, Pfizer Pty Ltd., Sydney, Australia.

[2] Crystalline Penicillin G, Evans Medical Australia (Pty) Ltd., Sydney, Australia.

[3] McIlwain's tissue chopper (7), Mickle Laboratory Engineering Co., Mill Works, Gomshall, Surrey, England.

[4] 1 part of 5% hydrogen peroxide to 9 parts of 0.556% benzidine in 12.5% acetic acid kept at 4°C.

[5] The following abbreviations are used: SRBC, sheep erythrocytes; PFC, hemolysin plaque-forming cells; AFCP, antibody-forming cell precursors; ARC, antigen-reactive cells; and SE, standard error.

mus cells did not increase the number of PFC above that obtained in irradiated mice given SRBC alone. Increasing the number of thymus cells to 50 million increased the average number of PFC to only 45. Injection of 25 million thoracic duct cells in the absence of antigen did not cause any elevation above the background number of 15 PFC per spleen. Similarly, bone marrow cells were ineffective.

During investigations of the hemolytic focus-producing capacity of thoracic duct and spleen cell inocula (2) it was noted that the foci produced by thoracic duct cells involved only one or two consecutive segments of the sliced spleens. With spleen cell inocula, hemopoietic regeneration occurred in the irradiated spleens and individual hemolytic foci often extended over three or four seg-

TABLE I

PFC Produced in the Spleens of Heavily Irradiated CBA Mice after Injection of SRBC and Syngeneic Thymus, Bone Marrow, or Thoracic Duct Cells

Cells inoculated	No. of mice	Average PFC per spleen 8 days postirradiation (± SE)
SRBC only	16	15 ± 6.1
10×10^6 thymus cells + SRBC	14	15 ± 3.0
50×10^6 thymus cells + SRBC	8	45 ± 5.0
10×10^6 bone marrow cells + SRBC	16	27 ± 5.8
25×10^6 thoracic duct cells only	5	17 ± 4.4
10×10^6 thoracic duct cells + SRBC	23	1270 ± 338*

* $P < 0.05 - < 0.01$ when this value is compared with those in all other groups.

ments. To investigate whatever effect hemopoietic regeneration might have on the size of hemolytic foci produced by thoracic duct cells, bone marrow cells were injected simultaneously. In Table II, the results of two experiments using (CBA × C57BL)F_1 mice as both donors and recipients, are shown. A mixed inoculum of one million thoracic duct cells and 10 million bone marrow cells did not increase the number of hemolytic foci per spleen over that produced by one million thoracic duct cells alone but greatly increased the number of PFC per spleen.

A time course study of the production of hemolytic foci and PFC per spleen was performed in heavily irradiated CBA mice injected with SRBC and one million CBA thoracic duct cells, 10 million CBA bone marrow cells or a mixed inoculum of both cell types. 4–9 days after irradiation and cell inoculation, half the number of spleens were assayed for their content of PFC and the other half were serially sectioned to determine the number of hemolytic foci. The results are shown in Figs. 1 and 2. The majority of heavily irradiated CBA mice of our colony had died by 9 days unless they were given hemopoietic

cells. Hence adequate data was not available at 9 days in the case of irradiated mice injected with SRBC and thoracic duct cells alone. At day 8, one million thoracic duct cells produced on the average about 100 PFC and five hemolytic foci per spleen. Addition of bone marrow cells to the inoculum of thoracic duct cells significantly increased the number of PFC per spleen to about 900 ($P < 0.01$) but did not increase the number of hemolytic foci. As the foci presumably represent discrete clusters of PFC, it can be calculated that, at the peak of the response, the foci resulting from inoculation of thoracic duct cells contain about 20 PFC. The addition of bone marrow cells increased this number to

TABLE II

Hemolytic Foci and PFC Produced in the Spleens of Heavily Irradiated (CBA × C57BL)F_1 Mice after Injection of SRBC and Syngeneic F_1 Thoracic Duct and F_1 Bone Marrow Cells

Cells inoculated	Hemolytic foci 8 days postirradiation		PFC 8 days post-irradiation	
	No. of mice tested	No. of foci per spleen	No. of mice tested	Average No. of PFC per spleen (± SE)
SRBC only	10	0, 0, 0, 0, 0, 0, 0, 1, 1, 1	8	12 ± 3.5
10×10^6 bone marrow cells + SRBC	4	0, 0, 0, 1	4	31 ± 2.9
10^6 thoracic duct cells + SRBC	12	3, 3, 3, 4, 5, 5, 5, 6, 6, 7, 8, 8	13	44 ± 12.3
10^6 thoracic duct cells + 10×10^6 bone marrow cells + SRBC	13	2, 2, 3, 3, 3, 6, 6, 6, 7, 7, 7, 7, 10	16	527 ± 75.4

approximately 160 PFC. The 7–9 day PFC response of irradiated CBA mice injected with 10 million CBA bone marrow cells varied from 27 ± 5.8 in one series of experiments (Table I) to 110 ± 21.1 in another (Fig. 1). The number of hemolytic foci in bone marrow–injected mice however never increased significantly above the background number of one hemolytic focus per spleen obtained in irradiated mice injected with either SRBC only or lymphoid cells only. These results indicate that while irradiated mice given SRBC and thoracic duct cells alone can produce PFC, this response is greatly increased by the simultaneous injection of bone marrow cells.

When thymus cells were used instead of thoracic duct cells, it was evident that even 50 million thymus cells failed to achieve the same effect as one million thoracic duct cells in irradiated mice injected with SRBC and bone marrow (Table III). Therefore thoracic duct cells were far superior to thymus cells in this system in contrast to what was observed in neonatally thymectomized mice (1). This finding prompted the following studies in adult thymectomized irradiated mice injected with bone marrow.

Effect of Syngeneic Thymus or Thoracic Duct Cells in Thymectomized Irradiated Bone Marrow-Protected Hosts.—10 million CBA thymus or thoracic duct cells were injected together with SRBC into adult thymectomized mice 2

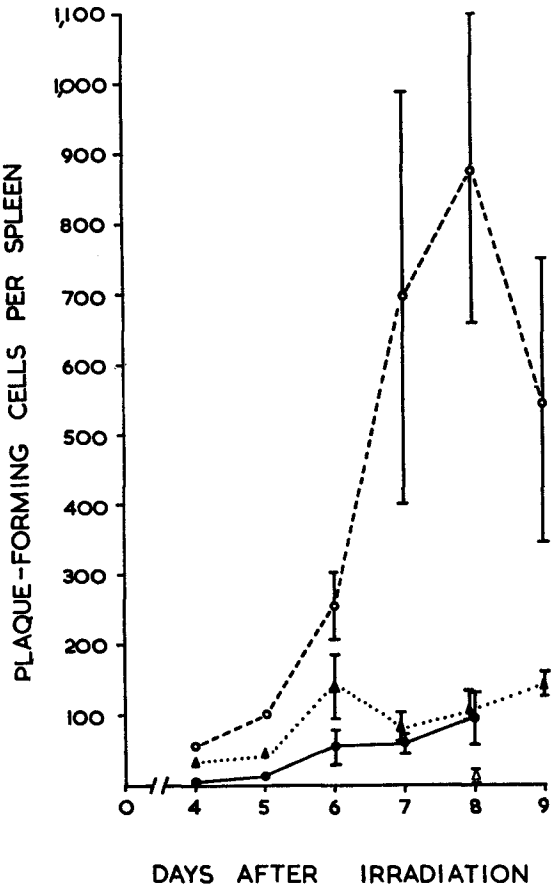

FIG. 1. PFC produced in the spleens of heavily irradiated CBA mice injected, after irradiation, with SRBC alone (△), SRBC and 1 million syngeneic thoracic duct cells (●——●), SRBC and 10 million syngeneic bone marrow cells (▲....▲), and SRBC and a mixed inoculum of 1 million syngeneic thoracic duct cells and 10 million syngeneic bone marrow cells (○——○). The magnitude of twice the standard errors is shown by the vertical bars. Each point at 4, 5, and 9 days represents the mean of determinations made on 2–5 mice and at 6, 7, and 8 days on 6–13 mice.

wk after irradiation and marrow protection. The number of PFC per spleen was determined 2, 4, 5, 7, and 10 days thereafter. From Fig. 3 it can be seen that while mice given thoracic duct cells produced 72,000 PFC, mice receiving the same number of thymus cells could produce only about 6000 PFC at the

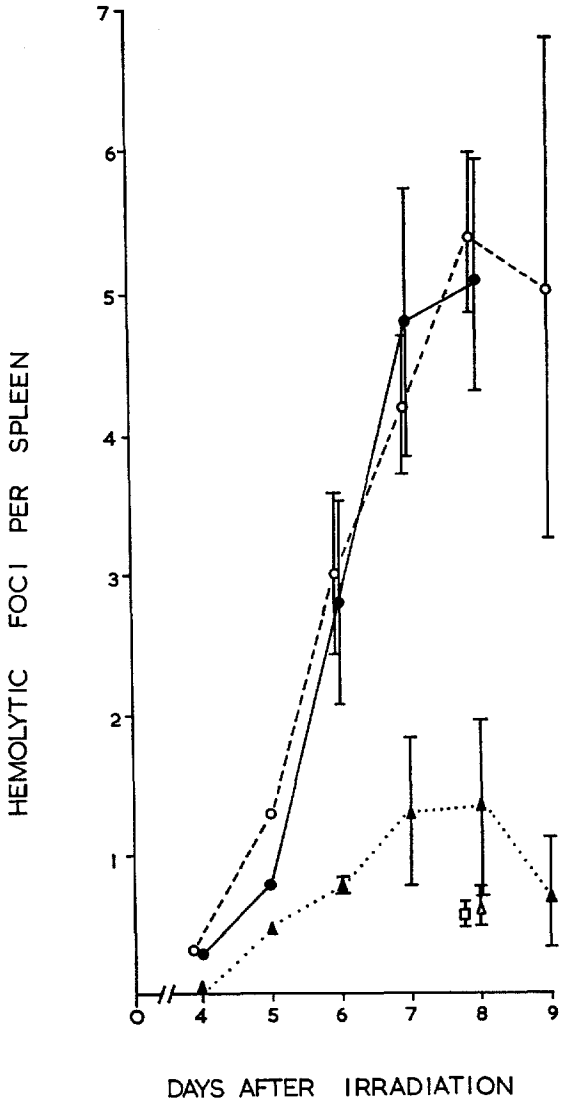

FIG. 2. Hemolytic foci produced in the spleens of heavily irradiated CBA mice injected after irradiation with SRBC alone (△), 1 million syngeneic thoracic duct cells alone (□), SRBC and 1 million syngeneic thoracic duct cells (●——●), SRBC and 10 million syngeneic bone marrow cells (▲....▲), and SRBC and a mixed inoculum of 1 million syngeneic thoracic duct cells and 10 million syngeneic bone marrow cells (○——○). The magnitude of twice the standard errors is shown by the vertical bars. Each point at 4, 5, and 9 days represents the mean of determinations made on 2–4 mice and at 6, 7, and 8 days on 6–14 mice.

height of the response. Mice injected with SRBC only gave less than 300 PFC. In the case of thoracic duct cell recipients, the number of PFC increased 12-fold between days 4 and 5. When the data for 10 million thoracic duct cells are compared in Table I and Fig. 3, it is evident that far more PFC were produced in the bone marrow–protected thymectomized irradiated hosts than in the irradiated hosts not given bone marrow.

It is apparent, therefore, that thymus cells are not as effective as thoracic duct cells in thymectomized irradiated bone marrow–protected hosts in contrast to the situation (1) in neonatally thymectomized mice. One notable difference between the two hosts was the size of their spleens. Thus, seven mice which had been thymectomized at 6 wk, irradiated and injected with

TABLE III

PFC Produced in the Spleens of Heavily Irradiated CBA Mice after Injection of SRBC and Syngeneic Thymus or Thoracic Duct Cells together with Bone Marrow Cells

Cells inoculated	No. of mice	Average PFC per spleen 8 days post-irradiation (± SE)
SRBC	12	13 ± 1.8
10×10^6 bone marrow cells + SRBC	15	73 ± 23.5
50×10^6 thymus cells + SRBC	20	52 ± 19.3
50×10^6 thymus cells + 10×10^6 bone marrow cells + SRBC	13	522 ± 341
10^6 thoracic duct cells + SRBC	10	97 ± 34.7
10^6 thoracic duct cells + 10×10^6 bone marrow cells + SRBC	13	877 ± 218

bone marrow cells had an average of 81 ± 7.0 million nucleated spleen cells 2 wk after irradiation, which is significantly less than the average number of 148 million cells in the spleens of neonatally thymectomized mice (1). The possibility that this accounts for the different results will be discussed later.

Effect of Allogeneic Thymus, Thoracic Duct, and Bone Marrow Cells in Irradiated Hosts.—In order to identify, by means of anti-H2 sera, the identity of PFC produced in irradiated hosts in response to SRBC and thymus, thoracic duct and bone marrow cells, allogeneic combinations must be used. Accordingly, (CBA × C57BL)F_1 thoracic duct cells were injected together with SRBC and CBA bone marrow into heavily irradiated CBA mice. The results of two representative experiments are shown in Table IV. When a mixed inoculum of F_1 thoracic duct cells, CBA bone marrow cells, and SRBC was given on the day of irradiation, 800 PFC appeared in the spleens 7 days later. This was only slightly greater than the sum of the mean number of PFC produced in the spleens of mice given either SRBC alone, SRBC and thoracic

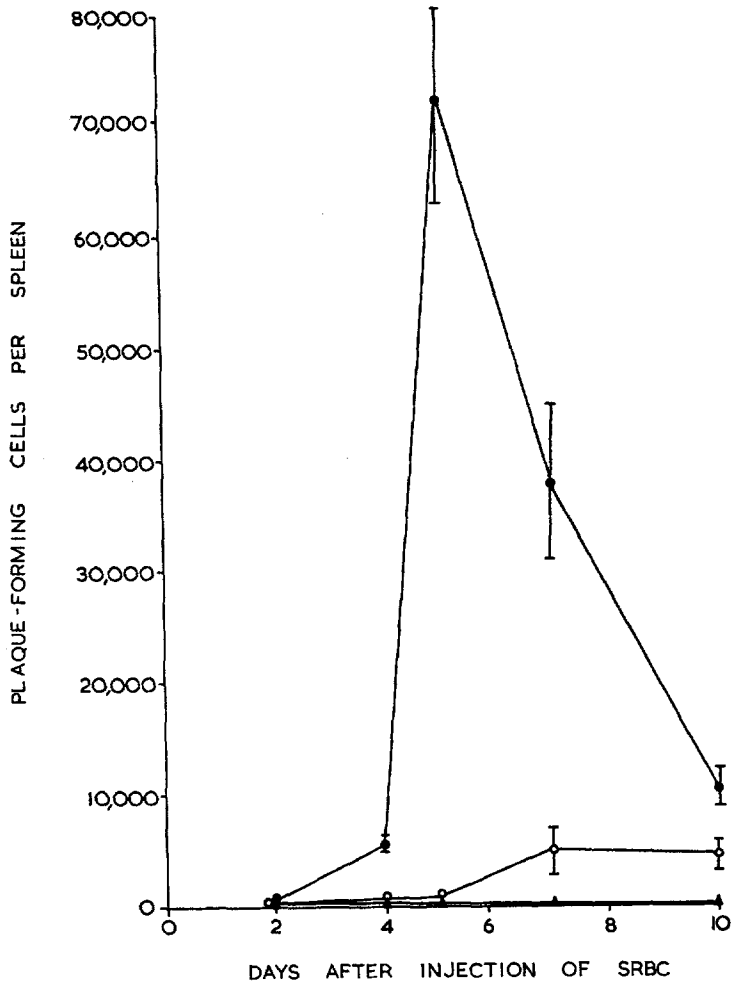

FIG. 3. PFC produced in the spleens of adult thymectomized CBA mice, heavily irradiated and protected with syngeneic bone marrow cells 2 wk previously, and injected with SRBC alone (▲——▲), SRBC and 10 million syngeneic thymus cells (○——○), and SRBC and 10 million syngeneic thoracic duct cells (●——●). The magnitude of twice the standard errors is shown by the vertical bars. Each point represents the mean of determinations made on 3–10 mice.

duct cells, or SRBC and bone marrow cells.[6] These results therefore contrast with those obtained when syngeneic combinations of cells were used (*vide supra*). Attempts were then made to determine whether synergism between

[6] Likewise, when irradiated F_1 mice were used, instead of CBA mice, as recipients of a mixed inoculum of SRBC, F_1 thoracic duct cells and CBA bone marrow, no convincing synergistic effect was obtained.

allogeneic cells could take place in irradiated hosts if CBA bone marrow cells were allowed to reside in their hosts for a period of time before introducing F_1 thoracic duct cells and antigen. As shown in Table IV, giving thoracic duct cells and SRBC 2 days after bone marrow did not obviously augment the PFC response. Increasing this interval still further was not attempted in these irradiated hosts since it is well known that immunological recovery takes place in irradiated mice given bone marrow. If adult mice are thymectomized prior to irradiation and bone marrow protection, however, the recovery of immunological capacity is impaired (8). This type of host was therefore chosen to deter-

TABLE IV

PFC Produced in the Spleens of Heavily Irradiated CBA Mice after Injection of SRBC, (CBA × C57BL)F_1 Thoracic Duct, and CBA Bone Marrow Cells

Exp.	Cells inoculated	No. of mice	Average PFC per spleen 7 days after SRBC
1	6×10^6 F_1 thoracic duct cells + 10×10^6 CBA bone marrow cells + SRBC	6	800
	6×10^6 F_1 thoracic duct cells + SRBC	3	593
	10×10^6 CBA bone marrow cells + SRBC	8	116
	SRBC only	10	19
2	10×10^6 CBA bone marrow cells followed 2 days later by 3×10^6 F_1 thoracic duct cells + SRBC	6	496
	10×10^6 CBA bone marrow cells followed 2 days later by SRBC	8	103
	3×10^6 F_1 thoracic duct cells + SRBC	8	228
	SRBC only	8	43

mine whether synergism between allogeneic cells can take place. 10 million F_1 thymus or thoracic duct cells were injected 2 wk after protecting irradiated adult thymectomized CBA mice with CBA bone marrow. The results of a time course study on the PFC responses of these mice are shown in Fig. 4. Thoracic duct cells increased the response to 19,800 PFC at the peak. Thymus cells failed to produce an increase. A comparison of the results obtained in Figs. 3 and 4 clearly shows that, in thymectomized irradiated bone marrow–protected hosts, syngeneic cells are more effective than semiallogeneic cells.

Identity of the Hemolysin Plaque-Forming Cells.—When spleen cell suspensions containing PFC were incubated with specific isoantisera the number of detectable PFC was reduced by 80–100%. Nonspecific isoantisera or normal mouse sera reduced the number to an insignificant extent (1).

Spleen cells from irradiated CBA mice given F_1 thoracic duct cells and SRBC on the day of irradiation were incubated with normal CBA or C57BL serum,

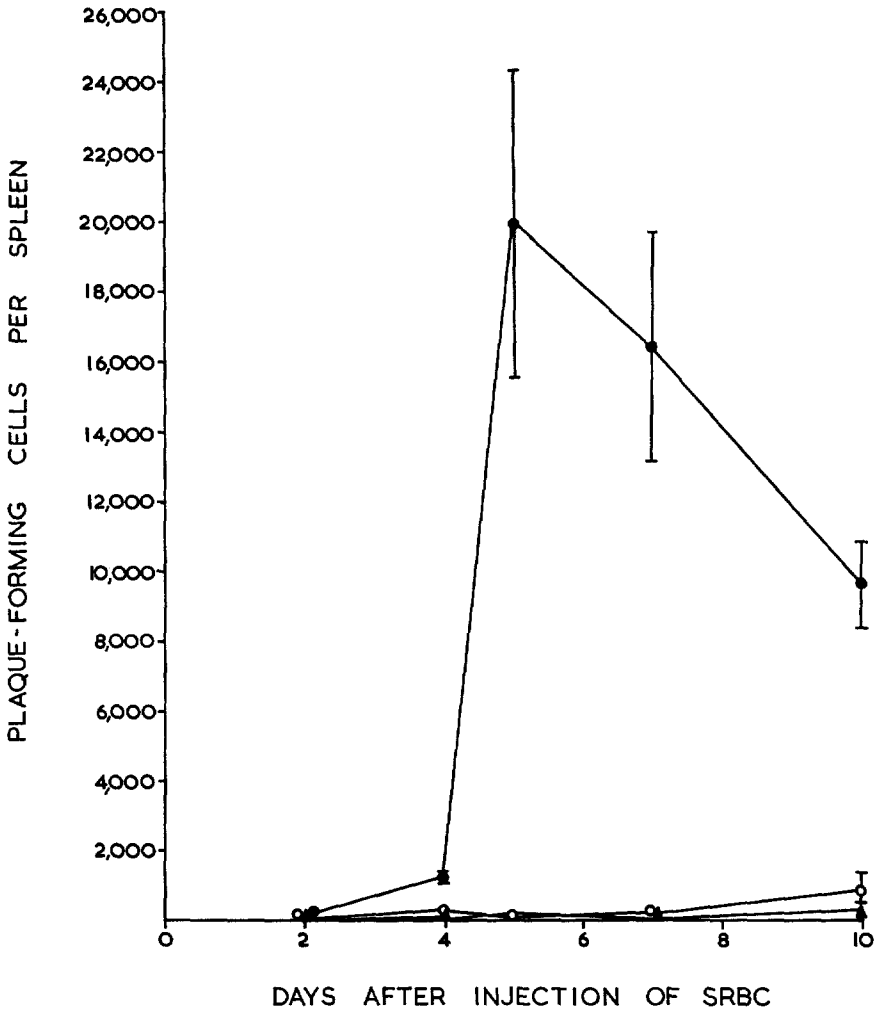

FIG. 4. PFC produced in the spleens of adult thymectomized CBA mice, heavily irradiated and protected with syngeneic bone marrow cells 2 wk previously, and injected with SRBC alone (▲——▲), SRBC and 10 million semiallogeneic (CBA × C57BL)F_1 thymus cells (○——○), and SRBC and 10 million semiallogeneic F_1 thoracic duct cells (●——●). The magnitude of twice the standard errors is shown by the vertical bars. Each point represents the mean of determinations made on 2–10 mice.

anti-CBA serum or anti-C57BL serum. The results of three experiments with 14 mice injected with various numbers of thoracic duct cells are shown in Table V. Anti-CBA serum reduced the number of PFC by 85–93% and anti-C57BL serum reduced the number by 85–95%. It is thus apparent that the PFC produced in these hosts were derived from the inoculated cells.

The number of PFC produced following an inoculation of F_1 thoracic duct cells and SRBC was far greater in adult thymectomized irradiated CBA mice injected with CBA bone marrow than in irradiated mice not receiving bone marrow (cf. Tables V and VI). In Table VI are shown the results of incubating

TABLE V

PFC from Spleens of Reconstituted Irradiated CBA Mice Remaining after Incubation with Isoantisera

Cells used for reconstitution	No. of spleens in pool	Average PFC per spleen at 7 days	No. of PFC per aliquot remaining after incubation with:		
			Normal mouse serum	Anti-CBA serum	Anti-C57BL serum
6×10^6 F_1 thoracic duct cells	3	593	98	14 (85%)*	11 (89%)
10×10^6 F_1 thoracic duct cells	7	822	72	6 (92%)	11 (85%)
30×10^6 F_1 thoracic duct cells	4	5,655	432	32 (93%)	21 (95%)

* Number in brackets refers to per cent reduction.

TABLE VI

PFC from Spleens of Reconstituted Thymectomized Irradiated CBA Mice Remaining after Incubation with Isoantisera

Cells used or reconstitution*	No. of spleens in pool	Average PFC per spleen	No. of PFC per aliquot remaining after incubation with:		
			Normal mouse serum	Anti-CBA serum	Anti-C57BL serum
10×10^6 F_1 thoracic duct cells	5	10,980	80	4 (95%)‡	88 (0%)
10×10^6 F_1 thoracic duct cells	5	18,262§	139	6 (96%)	140 (0%)
35×10^6 F_1 thoracic duct cells	2	69,300	604	20 (97%)	571 (5%)
35×10^6 F_1 thoracic duct cells	2	85,850	1454	70 (95%)	1660 (0%)

* F_1 thoracic duct cells were inoculated 2 wk postirradiation and marrow protection.

‡ Number in brackets refers to per cent reduction.

§ Spleens assayed 7 days after thoracic duct cells were inoculated; all others were assayed 5 days after inoculation.

with isoantisera, aliquots of spleen cells from thymectomized irradiated recipients of F_1 thoracic duct cells and CBA bone marrow. Anti-CBA serum caused a reduction in the number of PFC of 95–97% and anti-C57BL resulted in losses of up to 5%. The majority of PFC produced in these hosts was therefore derived not from inoculated F_1 thoracic duct cells but from CBA cells.

DISCUSSION

Cells capable of producing hemolytic foci in the spleens of heavily irradiated mice, above the background number, were detected in thoracic duct lympho-

cyte suspensions but not in bone marrow. In irradiated recipients of thoracic duct cells and SRBC, the addition of syngeneic bone marrow did not increase the number of hemolytic foci but increased the number of PFC per spleen to a far greater extent than could be accounted for by summating the activities of either cell population alone. These results can be interpreted in at least two ways. (*a*) By allowing hemopoietic regeneration and possibly creating a favorable environment in the spleen, bone marrow promotes the differentiation and multiplication of PFC precursors present in thoracic duct lymph. Such PFC precursors were described as antigen-sensitive cells by Kennedy et al. (5) who considered that the PFC clustered within an area detectable by the hemolytic focus assay technique, were the direct descendants of an antigen-sensitive cell that had lodged in the spleen at the site where the focus arose. As bone marrow did not produce foci, these investigators concluded that it lacked antigen sensitive cells or PFC precursors according to their definition. (*b*) The second possibility is that bone marrow does provide PFC precursors (the AFCP in our terminology) the differentiation of which can be induced only by the associated presence of some type of cell, presumably derived originally from the thymus and present in thoracic duct lymph (the ARC in our terminology). Clearly, in order to distinguish between these two possibilities, one must identify the source of PFC in irradiated mice receiving bone marrow and thoracic duct or thymus lymphocytes.

In the experiments of Davies et al. (9–11), thymectomized irradiated mice were protected with bone marrow and thymus from donors which were slightly different immunogenetically. When the spleens from these thymus-grafted mice were transferred soon after challenge with SRBC, into irradiated recipients presensitized against either the thymus-donor or the marrow-donor, those capable of rejecting cells of thymus donor-type were able to produce antibody in response to SRBC. By contrast, those immunized against marrow donor-type cells produced much less antibody. These transfer experiments were, however, performed 30 days after irradiation and thymus grafting. It is known that, at that time, the lymphoid cell population of the graft has been replaced entirely by cells derived from the bone marrow (12). Thus hemolysins detected in irradiated mice, presensitized against thymus donor-type cells, may have been produced by bone marrow-derived cells which had repopulated, and migrated from, the thymus graft. Hence the cells producing antibody could have the immunogenetic characteristic of the marrow donor and yet be thymus derived. Thus, "it may be that thymus-derived cells can produce antibody, but only in the presence of cells of bone marrow origin. Equally, cells of bone marrow origin . . . may be the cells whose immunological potential is enhanced by association with cells of thymic origin. These are not problems which the present analysis can resolve" (11).

In the present study, no attempt was made to determine, with anti-H2 sera,

the identity of the PFC produced in the spleens of irradiated recipients of a *mixed* inoculum of F_1 thoracic duct lymphocytes, CBA bone marrow cells, and SRBC, because no synergistic effect was observed in this experimental system. This contrasts with the results obtained in the syngeneic situation but is in general agreement with a previous report indicating that allogeneic thymus and bone marrow cells do not interact in irradiated hosts (13). Since it appears that a synergistic effect can be obtained only in syngeneic systems, methods other than those using anti-H2 sera must be devised to determine the source of the PFC. The only technique available so far is the detection of a particular chromosome marker in individual antibody-forming cells (14). This has been applied to PFC obtained from the spleens of heavily irradiated mice injected simultaneously after irradiation with CBA/T6T6 bone marrow cells, CBA thoracic duct cells, and SRBC and the results will be given in the following paper (15).

The reason for the failure of an interaction to take place between allogeneic lymphocytes and bone marrow cells immediately following transfer to irradiated hosts, is unknown but it may be that the phenomenon of allogeneic inhibition (16) operates. If this is so, however, it no longer operates once differentiation of some bone marrow stem cell has taken place, since an excellent synergistic effect was obtained in thymectomized irradiated mice receiving F_1 thoracic duct cells and SRBC 2 wk after an injection of CBA bone marrow. In this system, therefore, techniques using anti-H2 sera were used to determine the identity of the PFC produced. The results indicate that the bone marrow must be a source of AFCP since the PFC were CBA-type and not F_1-type. This conclusion is corroborated by the results obtained in the following study in which chromosome marker techniques were used (15). It seems therefore that bone marrow provides AFCP, but not cells capable of *initiating* the production of hemolytic foci, so that it must lack ARC. The hemolytic focus assay is thus a measure, not of the number of PFC precursors in a given population, but of the number of ARC which settle in the irradiated spleens.

Irradiated recipients of thymus cells and SRBC will produce neither hemolytic foci nor PFC in their spleens (5, 17–19). When bone marrow is added, however, both hemolytic foci and an increase in the number of PFC per spleens can be detected (17–19). One of the differences between the population of cells from thymus and thoracic duct lymph, which could not have been demonstrated in the experimental system used in the previous study (1), is that PFC can be derived directly from some thoracic duct lymphocytes, but not from thymus cells. The evidence for this is that PFC were produced in the spleens of heavily irradiated recipients of syngeneic thoracic duct cells but not of thymus cells, and that the PFC in the spleens of irradiated CBA mice injected with F_1 thoracic duct cells were F_1 type. All the evidence obtained in this and the previous study therefore suggests that the thymus contains only ARC, the

bone marrow only AFCP, but the thoracic duct lymph contains both ARC and AFCP. Preliminary studies using density gradient centrifugation techniques indicate that the two cell types in thoracic duct lymph may indeed be separated.

It is evident from a lot of experimental data that the immunological activity of thymus cells is inferior to that of spleen or lymph node cells (20). For instance, mouse thymus cells injected with rat erythrocytes into preirradiated mice enable their hosts to produce much less antibody than a similar number of spleen cells (21). Furthermore, in order to prevent the protective action of allogeneic bone marrow in irradiated mice two to four times as many syngeneic thymus lymphocytes as syngeneic lymph node cells were required (22, 23). In the present experiments, a comparison was made between the peak PFC response of irradiated recipients of thymus and bone marrow on the one hand, and of thoracic duct cells and bone marrow on the other. Far more thymus than thoracic duct lymphocytes were required to cooperate with a certain number of bone marrow cells in order to produce a given number of PFC. The mean PFC response in irradiated recipients of thymus and bone marrow was greater than that of mice given either cell population alone, but there was a considerable variation between individual mice (Table III). The synergistic effect in these experiments was much less impressive than that reported by Claman et al. (17), perhaps because they gave two injections of SRBC to their irradiated recipients whereas only one such injection was given here at the time of cell transfer. Thymus cells were also less effective than thoracic duct cells in thymectomized irradiated, bone marrow–protected mice. This difference between the two cell types was not evident in neonatally thymectomized recipients (1). The spleens of these mice were much larger than those of thymectomized irradiated mice. It is possible that the larger spleens trap more thymus cells since these cells preferentially home there rather than to lymph nodes (24, 25). Thoracic duct cells, however, have a capacity to recirculate and to home to all the lymphoid tissues (26). These features might account for the different results obtained in neonatally thymectomized and in adult thymectomized irradiated, bone marrow–protected mice. An alternative possibility is that a particular cell type, which is radiosensitive and required for antigen handling in this system, is present in spleen and thoracic duct lymph, but absent from the thymus lymphocyte population. Neonatal thymectomy would presumably not affect this cell type.

As a general conclusion, it may be said that there are ARC in the thymus but in a proportion that is much less than that found in the cell population of thoracic duct lymph, spleen, and lymph nodes. Perhaps ARC are exported out of the thymus as soon as they are formed so that the thymus would contain a greater proportion of "immature" cells. Perhaps emigrating thymus cells must undergo a period of maturation before they can become ARC. Perhaps

this maturation entails interaction with antigen (or macrophage-processed antigen) to give rise to a clone of cells that are specifically reactive to the particular antigen (? memory cells). Perhaps only these cells are capable of recirculating so that the population of cells in thoracic duct lymph would be richer in ARC than that in the thymus. Whatever the case may be, it seems evident that in the 19S immune response to SRBC, some interaction takes place between thymus-derived ARC and bone marrow-derived AFCP and that this allows the differentiation of the AFCP to PFC. Whether a similar interaction takes place in other antigenic systems is not known but it would seem more likely to occur only in those immune responses which are initiated by cells the development of which is under thymus control.

SUMMARY

The number of discrete hemolytic foci and of hemolysin-forming cells arising in the spleens of heavily irradiated mice given sheep erythrocytes and either syngeneic thymus or bone marrow was not significantly greater than that detected in controls given antigen alone. Thoracic duct cells injected with sheep erythrocytes significantly increased the number of hemolytic foci and 10 million cells gave rise to over 1000 hemolysin-forming cells per spleen. A synergistic effect was observed when syngeneic thoracic duct cells were mixed with syngeneic marrow cells: the number of hemolysin-forming cells produced in this case was far greater than could be accounted for by summating the activities of either cell population given alone. The number of hemolytic foci produced by the mixed population was not however greater than that produced by an equivalent number of thoracic duct cells given without bone marrow. Thymus cells given together with syngeneic bone marrow enabled irradiated mice to produce hemolysin-forming cells but were much less effective than the same number of thoracic duct cells. Likewise syngeneic thymus cells were not as effective as thoracic duct cells in enabling thymectomized irradiated bone marrow–protected hosts to produce hemolysin-forming cells in response to sheep erythrocytes.

Irradiated recipients of semiallogeneic thoracic duct cells produced hemolysin-forming cells of donor-type as shown by the use of anti-H2 sera. The identity of the hemolysin-forming cells in the spleens of irradiated mice receiving a mixed inoculum of semiallogeneic thoracic duct cells and syngeneic marrow was not determined because no synergistic effect was obtained in these recipients in contrast to the results in the syngeneic situation. Thymectomized irradiated mice protected with bone marrow for a period of 2 wk and injected with semiallogeneic thoracic duct cells together with sheep erythrocytes did however produce a far greater number of hemolysin-forming cells than irradiated mice receiving the same number of thoracic duct cells without bone marrow. Anti-H2 sera revealed that the antibody-forming cells arising in the

spleens of these thymectomized irradiated hosts were derived, not from the injected thoracic duct cells, but from bone marrow.

It is concluded that thoracic duct lymph contains a mixture of cell types: some are hemolysin-forming cell precursors and others are antigen-reactive cells which can interact with antigen and initiate the differentiation of hemolysin-forming cell precursors to antibody-forming cells. Bone marrow contains only precursors of hemolysin-forming cells and thymus contains only antigen-reactive cells but in a proportion that is far less than in thoracic duct lymph.

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BIBLIOGRAPHY

1. Miller, J. F. A. P., and G. F. Mitchell. 1968. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J. Exptl. Med.* **128**:801.
2. Miller, J. F. A. P., G. F. Mitchell, and N. S. Weiss. 1967. Cellular basis of the immunological defects in thymectomized mice. *Nature.* **214**:992.
3. Miller, J. F. A. P. 1960. Studies on mouse leukaemia. The role of the thymus in leukaemogenesis by cell-free leukaemic filtrates. *Brit. J. Cancer.* **14**:93.
4. Jerne, N. K., A. A. Nordin, and C. Henry. 1963. The agar plaque technique for recognizing antibody-producing cells. *In* Cell Bound Antibodies. B. Amos and H. Koprowski, editors. Wistar Institute Press, Philadelphia. 109.
5. Kennedy, J. C., L. Siminovitch, J. E. Till, and E. A. McCulloch. 1965. A transplantation assay for mouse cells responsive to antigenic stimulation by sheep erythrocytes. *Proc. Soc. Exptl. Biol. Med.* **120**:863.
6. Playfair, J. H. L., B. W. Papermaster, and L. J. Cole. 1965. Focal antibody production by transferred spleen cells in irradiated mice. *Science.* **149**:998.
7. McIlwain, H., and H. L. Buddle. 1953. Techniques in tissue metabolism. I. A mechanical chopper. *Biochem. J.* **53**:412.
8. Cross, A. M., E. Leuchars, and J. F. A. P. Miller. 1964. Studies on the recovery of the immune response in irradiated mice thymectomized in adult life. *J. Exptl. Med.* **119**:837.
9. Davies, A. J. S., E. Leuchars, V. Wallis, and P. C. Koller. 1966. The mitotic response of thymus-derived cells to antigenic stimulus. *Transplantation.* **4**:438.
10. Davies, A. J. S., E. Leuchars, V. Wallis, R. Marchant, and E. V. Elliott. 1967. The failure of thymus-derived cells to produce antibody. *Transplantation.* **5**:222.
11. Davies, A. J. S., E. Leuchars, V. Wallis, N. R. S. C. Sinclair, and E. V. Elliott. 1968. The selective transfer test. An analysis of the primary response to sheep red cells. *In* Advance in Transplantation. J. Dausset, J. Hamburger, and G. Mathe, editors. Munksgaard, Copenhagen. 97.
12. Dukor, P., J. F. A. P. Miller, W. House, and V. Allman. 1965. Regeneration of thymus grafts. I. Histological and cytological aspects. *Transplantation.* **3**:639.
13. Chaperon, E. A., and H. N. Claman. 1967. Effect of histocompatibility differences on the plaque-forming potential of transferred lymphoid cells. *Federation Proc.* **26**:640.

14. Nossal, G. J. V., K. D. Shortman, J. F. A. P. Miller, G. F. Mitchell, and J. S. Haskill. 1967. The target cell in the induction of immunity and tolerance. *Cold Spring Harbor Symp. Quant. Biol.* **32**:369.
15. Nossal, G. J. V., A. Cunningham, G. F. Mitchell, and J. F. A. P. Miller. 1968. Cell to cell interaction in the immune response. III. Chromosomal marker analysis of single antibody-forming cells in reconstituted, irradiated, or thymectomized mice. *J. Exptl. Med.* **128**:839.
16. Möller, G., and E. Möller. 1966. Interaction between allogeneic cells in tissue transplantation. *Ann. N.Y. Acad. Sci.* **129**:735.
17. Claman, H. N., E. A. Chaperon, and R. F. Triplett. 1966. Thymus-marrow cell combinations—synergism in antibody production. *Proc. Soc. Exptl. Biol. Med.* **122**:1167.
18. Miller, J. F. A. P., and G. F. Mitchell. 1967. The thymus and the precursors of antigen reactive cells. *Nature.* **216**:659.
19. Mitchell, G. F., and J. F. A. P. Miller. 1968. Immunological activity of thymus and thoracic duct lymphocytes. *Proc. Natl. Acad. Sci. U. S.* **59**:296.
20. Miller, J. F. A. P., and D. Osoba. 1967. Current concepts of the immunological functions of the thymus. *Physiol. Rev.* **47**:437.
21. Thorbecke, G. J., and M. W. Cohen. 1964. Immunological competence and responsiveness of the thymus. *In* The Thymus. V. Defendi and D. Metcalf, editors. Wistar Institute Press, Philadelphia. 33.
22. Vos, O., M. J. de Vries, J. S. Collenteur, and D. W. van Bekkum. 1959. Transplantation of homologous and heterologous lymphoid cells in x-irradiated and non-irradiated mice. *J. Natl. Cancer Inst.* **23**:53.
23. Congdon, C. C., and D. B. Duda. 1961. Prevention of bone marrow heterografting. Use of isologous thymus in lethally irradiated mice. *Arch. Pathol.* **71**:311.
24. Fichtelius, K. E., and B. J. Bryant. 1964. On the fate of thymocytes. *In* The Thymus in Immunobiology. R. A. Good and A. E. Gabrielsen, editors. Harper and Row, New York. 274.
25. Parrott, D. M. V., M. A. B. de Sousa, and J. East. 1966. Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. *J. Exptl. Med.* **123**: 191.
26. Gowans, J. L., and E. J. Knight. 1964. The route of re-circulation of lymphocytes in the rat. *Proc. Roy. Soc. London, Ser. B.* **159**:257.