ANTIGEN-REACTIVE CELLS IN NORMAL, IMMUNIZED, AND TOLERANT MICE*,‡

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The direct assessment of the antigen-reactive cell (ARC)[1] in most lymphoid tissues has, to date, been limited to the heterologous erythrocyte antigens (1–5). Because of the limitations implicit in the nature of these antigens regarding the secondary response and tolerance, a method has been devised to enumerate the ARC using the purified protein antigen, polymerized flagellin of *Salmonella adelaide* (POL).

In the preceding paper (6), the relevant technique has been described and it has been shown that, using this method, the foci of bacterial inhibition produced by the progeny of ARC in spleens of lethally irradiated syngeneic host mice, 8 days after receiving normal donor spleen cells and antigen, are antibody dependent and contain antibody-forming cells (AFC). The linear relationship between the number of foci and number of injected spleen cells, within a limited cell injection range, indicates that each focus is the response of one ARC.

In this paper, we have employed the described method for the enumeration of ARC in three areas of immunological interest. The first aspect is the study of lymphoid tissues generally, including bone marrow, thymus, and thoracic duct lymphocytes (TDL). Secondly, a study of the kinetics of the ARC in the early stages of the primary response and the related AFC response is reported. Thirdly, the kinetics of ARC in tolerance and the early posttolerance state is described.

From this work there is evidence that POL is able to stimulate ARC in bone marrow without the presence of thymus cells, as opposed to experience using the

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[1] The following abbreviations are used in this paper: ARC, antigen-reactive cell; POL, polymer antigen of *S. adelaide* flagellin; AFC, antibody-forming cell; TDL, thoracic duct lymphocytes; SRBC, sheep red blood cell; MON, monomer of *S. adelaide* flagellin; SA, specific activity; and PFC plaque-forming cells.

sheep red blood cell (SRBC) antigen (4, 5) and, as suspected, the lesion of tolerance appears to be at the level of the ARC or its precursors. Evidence is also presented to show that the number of ARC decreases to a level lower than that of unprimed spleen cell populations in the early primary response before reaching four to five times the normal level by the 6th postantigen day.

Materials and Methods

Animals.—Inbred CBA_{T6T6} and $C_{57}BL/Brad$ mice used in this study were 10–20 wk of age, fed on Barastoc dog cubes, cabbage, powdered milk, and tap water, and were housed in groups of five or less after irradiation. $C_{57}BL/Brad$ mice only were used for the tolerance studies. Since there appeared to be no sexual differences in the immune response, both males and females were used. Prior to all intravenous injections, mice were kept at 37°C for 15–30 min to produce dilatation of tail veins.

Irradiation.—Mice were irradiated using a Philips (R.T. 250) 250 kev machine, at 15 ma and 0.8 mm Cu HVL. The CBA_{T6T6} mice received 800 rads and the $C_{57}BL/Brad$ 625 rads in these experiments.

Antigen.—The polymer (POL) was prepared from flagella of *S. adelaide* (strain SW 1338, H antigen fg; O antigen 35) as described by Ada et al. (7). All dilutions of POL were made in 0.85% NaCl, stored frozen, and injected intravenously using the lateral tail vein. The cyanogen bromide digest (digest) of *S. adelaide* SW 1338 flagellin was used for the induction of immunological tolerance. This preparation, which retains the antigenic determinants of the intact flagellin molecule but not its immunogenicity, was prepared by the method of Parish et al. (8). To produce tolerance each animal received 10 μg of antigen intraperitoneally 6 days a week, commencing on the 1st day of life.

The monomer of *S. adelaide* flagellin (MON) was made up as previously described (7) and injected intravenously within 1 hr of preparation.

Serum Titrations.—For the routine collection of serum samples from tolerant animals, blood was obtained from the venous plexus of the eye under light ether anesthesia. All sera were separated from the blood clot within 24 hr, inactivated at 56°C for 30 min, frozen, and stored at −20°C for future titration. Doubling dilutions of serum were titrated using the bacterial immobilization technique. (9).

Bacteria.—The bacterial strain used as indicator in this study was *Salmonella derby* (strain SW 721, H antigen fg; O antigen 1, 4, 12, which shares the H but not O antigen with *S. adelaide*). On the day of assay, subcultures of *S. derby* were made from stock plates containing bacteria on semisolid, nutrient gelatine agar. A large enough cube of agar and bacteria from the stock plate was placed in the bacterial nutrient broth, so that by 2 hr of incubation at 37°C, the optical density of the solution at wave length 600 mμ was 78% transmittance, or approximately 5×10^7 organisms/ml.

Cells.—Cells for injection were prepared using aseptic technique. Spleens were minced and gently forced through a stainless steel sieve into a solution of 30% fetal calf serum in Eisen's balanced salt solution. The suspension was allowed to stand for 5 min at 4°C, the supernatant removed, the cells diluted to the appropriate concentration and counted using a hemocytometer before injection.

Thoracic duct lymphocytes (TDL) were obtained from normal CBA_{T6T6} mice using the method of Boak and Woodruff (10), collected in Dulbecco's medium containing 100 units of preservative-free heparin/ml and kept near 4°C. Before injecting, the cells were washed, resuspended in 30% fetal calf serum in Eisen's balanced salt solution, and counted using a hemocytometer.

Assay Method for ARC.—The details of this method have been described in the previous

paper (6). Briefly, lethally irradiated animals were injected with donor cells and 24 hr later challenged with 25 μg of POL. The animals were killed 8 days postirradiation, their spleen cut into slices 260 μ thick, and these were placed on a slide coated with agar containing the indicator strain of bacteria *S. derby*. After incubation, the slides were read for foci of antibody production. An ARC focus may be defined as one or more contiguous positive slices separated from other positive areas by at least one negative slice.

In some cases all slices were positive (confluence) and this was believed to be a result of ARC seeding so closely to each other that an overlapping of response occurred. The results of these spleens were included only in the determination of "specific activity" (SA). Claman et al. (4) used this calculation to indicate the percentage of slices within the spleen that were positive and found the SA to be linearly related to the number of foci. This measurement permits the quantitative scoring of a larger range of responses.

Statistically significant differences between responses in the tested cell populations were calculated using the nonparametric rank test.

TABLE I

Antigen-Reactive Cells in Cell Populations from Different Lymphoid Organs in CBA$_{T6T6}$ Mice

Cell source (and No. of animals tested)	No. of cells injected	Mean per cent specific activity	Mean ARC foci/spleen	Mean ARC foci/10^6 injected cells
Spleen (15)	2×10^6	33	2.5	1.2
Mesenteric lymph nodes (11)	1×10^6	43	2.2	2.2
Peyer's patches (10)	1×10^6	67	2.6	2.6
Thymus (11)	50×10^6	5	0.5	0.01
Bone marrow (7)	10×10^6	41	3	0.3
Bone marrow plus thymus (8)	50×10^6 plus 50×10^6	15	1.6	0.02

Background was 0.1 foci/spleen.

AFC Assay.—The AFC were enumerated using the adherence colony technique described by Diener (11). This detects AFC employing bacterial immunocytoadherence, a sensitive and specific assay in this system. Cells containing antibody are known to cause the adherence of numerous bacteria to their surface and when incubated in agar, a corona of bacteria around a cell will grow into a large colony which may be counted.

RESULTS

The Enumeration of ARC in Lymphoid Tissues.—Previous reports by other workers have quantitated directly the ARC in spleen (1, 2), lymph nodes (3), bone marrow and thymus (4), and TDL (5), using the SRBC antigen. To date, only ARC of the spleen have been enumerated using the POL antigen (6) leaving much of the lymphoid system unexplored. The need for assessing the status of the ARC in other lymphoid tissues than the spleen using POL therefore arises, not only to compare the result of the two antigen systems, but to extend the observations to the lymphoid cells associated with the gut.

Intestinal lymphoid tissue: It is generally believed that one of the major concentrations of lymphoid cells in the body is gut associated. In order to assess

the numbers of AFC precursors in at least a portion of this tissue, Peyer's patches were dissected off the gut wall and the mesenteric nodes removed and cell suspensions made of each tissue source for assay.

The proportion of ARC detected in these cell suspensions from CBA_{T6T6} mice, as recorded in Table I, were about twice that found in the spleen cell population. The SA, which gives an indication of the degree of immunological activity of the injected gut associated lymphoid cells, was found to be two times greater for gut associated cells than for spleen cells, which parallels the ARC response.

It has been shown that there may be differences in the levels of ARC in different strains of mice using the POL antigen system. For this reason, experiments were done on $C_{57}BL/Brad$ mice comparing the response of cells from Peyer's patches with those of spleen and of mesenteric lymph nodes. The re-

TABLE II

Numbers of Antigen-Reactive Cells in Cell Populations from Lymphoid Organs in $C_{57}BL$ Mice

Cell source (and No. of animals tested)	No of cells injected	Mean ARC per cent specific activity	Mean ARC foci/spleen	Mean ARC foci expressed per 10×10^6 cells injected
Spleen (9)	10×10^6	35	2.0	2.0
Mesenteric lymph nodes (11)	4.7×10^6	10	0.8	1.7
Peyer's patches (4)	4×10^6	40	1.0	2.5

Background, 0 foci per spleen.

sponse of ARC and SA in Peyer's patches was somewhat increased over that of spleen cells and mesenteric lymph node cells, although much less than in the CBA_{T6T6} response (Table II). However, the response of mesenteric lymph node cells was slightly less than that of spleen cells; the reverse of the situation seen in CBA_{T6T6} mice. Thus the observation that $C_{57}BL/Brad$ spleen cells were five times less responsive than CBA_{T6T6} spleen cells (6) was confirmed and extended to the cells from Peyer's patches and mesenteric lymph nodes as well.

Bone marrow and thymus: Recent work in the area of bone marrow–thymus interaction using the SRBC antigen indicates that thymus cells are necessary to enable bone marrow cells to give an optimal immune response in the adoptive transfer system (4, 5). Although neonatal thymectomy in mice produces an inability to respond to SRBC antigens, Humphrey et al. (12) have shown that neonatal thymectomy does not alter the immune response to all antigens in the same way. It is therefore of interest to know whether or not bone marrow cells require the presence of thymus cells before they are able to respond to POL. Employing a single intravenous POL challenge to stimulate transferred bone marrow cells in the irradiated host, there was a response of 3 ARC/10×10^6 injected cells and 41% SA (Table I). However, 50×10^6

thymus cells tested separately under similar circumstances gave only a background ARC and SA response, and when combined with 50×10^6 bone marrow cells a response was detected, although somewhat depressed as compared with 10×10^6 bone marrow cells alone. The marked decrease in the number of ARC foci when 50×10^6 bone marrow plus 50×10^6 thymus cells are injected remains unexplained. The difference could be related to fewer bone marrow cells seeding in the spleen because of the presence of large numbers of potentially competing thymus cells. Also the lack of increased numbers of ARC indicates that the handling of the immune response to POL is probably quite different from that of SRBC in the mouse.

Thoracic duct lymphocytes and bone marrow: The ARC content of TDL

TABLE III

The Numbers of ARC Foci and Their Activity in Populations of Bone Marrow and Thoracic Duct Lymphocytes

Cell type (and No. of animals tested)	Cell No. injected	Mean per cent specific activity	Mean ARC foci/spleen	Mean ARC foci/10^6 cells injected
TDL (10)	2×10^6	17	1.1	0.6
Postcolumn* TDL(7)	2×10^6	2	0.3	0.2
BM(7)	10×10^6	41	3.0	0.3
BM	10×10^6			
+	+			
TDL(6)	2×10^6	84	2.4	0.2

TDL, thoracic duct lymphocytes; BM, bone marrow cells.

* Postcolumn TDL. These cells were passed through a column of glass beads (50–70 μ) to obtain a population of about 99% small lymphocytes.

populations using the SRBC antigen has been shown to be about four times higher than that of normal spleen cells. In contrast, the ARC and SA response of TDL in the POL system was half that of spleen cell populations (Table III). Other TDL were passed through columns of small (50–70 μ) tightly packed siliconed glass beads, which effectively remove large and medium lymphocytes, leaving a filtrate of small lymphocytes (13). Contrary to findings using the SRBC antigen (14), when the postcolumn TDL (small lymphocytes) were injected into the irradiated host mice, the response was eight times less than that of normal spleen cells and about one-third of the unseparated TDL population.

Although bone marrow alone may respond in the POL system, it is of considerable interest to know whether or not circulating lymphocytes (TDL) would in any way enhance the response of bone marrow. Therefore, bone marrow cells were injected into irradiated hosts with TDL and the response in terms of ARC

was slightly lower than that of equivalent numbers of bone marrow cells alone. However, the response of the TDL–bone marrow combination in terms of SA was considerably more than the summation of the responses of the two cell populations tested separately, suggesting greater immunological activity in each ARC focus when the two cell types were combined.

The Kinetics of ARC and AFC in the Primary Response.—Since the ARC focus technique detects both IgM and IgG antibodies (6) it is possible to measure for the first time the total response in primed animals as well as those animals which have received a second challenge of antigen. The secondary response is believed by some to be the result of the response of both unprimed ARC and memory cells to the antigenic stimulus (15). By comparing the number of ARC detected after a primary injection with the numbers found in unprimed spleen cell populations, we should be able to obtain some clues pertaining to the kinetics of the memory cell. With this in mind, the short term effects of a priming dose of antigen at the level of the ARC in the CBA_{T6T6} mouse spleen were studied.

Donor animals were injected with POL, killed on days 1–4 and 6 days after challenge, and a sample of the spleen cells was tested for the number of AFC/10^6 cells. The remaining spleen cells from each time point were injected into an irradiated host, followed by 25 μg of POL 24 hr later. After 7 days, the mice were divided into two groups; one was used for the ARC focus assay of the spleen, and the other group was used to determine the number of AFC in the spleen.

Antigen concentrations used for priming spleen cells: It was shown previously that when increasing concentrations of antigen are injected into host mice which were lethally irradiated and injected with 2×10^6 normal spleen cells, the numbers of ARC increased to an optimal level (6). In those experiments, 25 μg of POL produced the optimal number of ARC, i.e., 2.5 ARC/spleen as a saturation dose and 5 μg of POL gave an easily detected but diminished number of 1.5 ARC/spleen.

Further, it is known that a better secondary response can be obtained in rats if the priming dose of POL stimulates a suboptimal response and is lower than the dose of the secondary challenge (16). For these reasons, two doses of antigen were chosen for priming the donor mice; 5 μg of POL which stimulates ARC focus activity suboptimally, and 25 μg of POL which appears to stimulate ARC activity maximally.

Trapping efficiency by host spleen of primed donor cells: In view of the possible different seeding rates of dividing cells (17), an experiment was done to determine whether or not antigenically stimulated donor spleen cells were trapped in the host spleen at a different rate than unstimulated cells 1–2 days postpriming with 25 μg of POL. Using the method described by Kennedy et al. (3) it was found that 8.5% of cells from mice 1 day after priming seeded in the

irradiated host spleen, which is only slightly different from the seeding efficiency of 5% found for unprimed spleen cells (6). Consequently, this increase in the efficiency of seeding in the spleen is not considered enough to make any significant difference in the overall results.

Kinetics of the AFC in primed donor spleen cells before transfer: The numbers of AFC present in animals primed with 25 μg of POL ranged from 5 AFC/10^6 on day 1 to a peak of 1030 AFC/10^6 on day 3 followed by a rapid

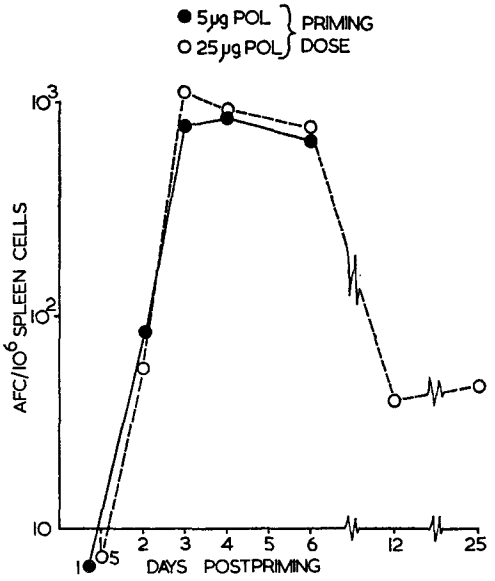

FIG. 1. The AFC response in CBA_{T6T6} mice after priming with POL. All points represent the mean number of AFC detected per 1×10^6 spleen cells and are the result of from four to eight test mice, except in the case of day 12 and 25 where each point represents two animals.

decline to about 40 AFC/10^6 injected cells by the 12th postantigen day (Fig. 1). The rise in AFC from about 60/10^6 to 1030/10^6 spleen cells between days 2 and 3 indicates that the doubling time for AFC was 6 hr, which is somewhat short and therefore may be due to other processes, such as precursor cell differentiation, as well as cell division. (18).

Kinetics of ARC during the primary response: For the sake of convenience and clarity, primed donor spleen cells injected into lethally irradiated host mice which were subsequently challenged 24 hr later with 25 μg of POL, will be described according to the priming dose of antigen and the number of days between priming and cell transfer. Thus, cells primed with 25 μg of POL and transferred on day 1 will be referred to as "25 μg–1 cells."

When compared with results obtained from unprimed donor spleen cells, the

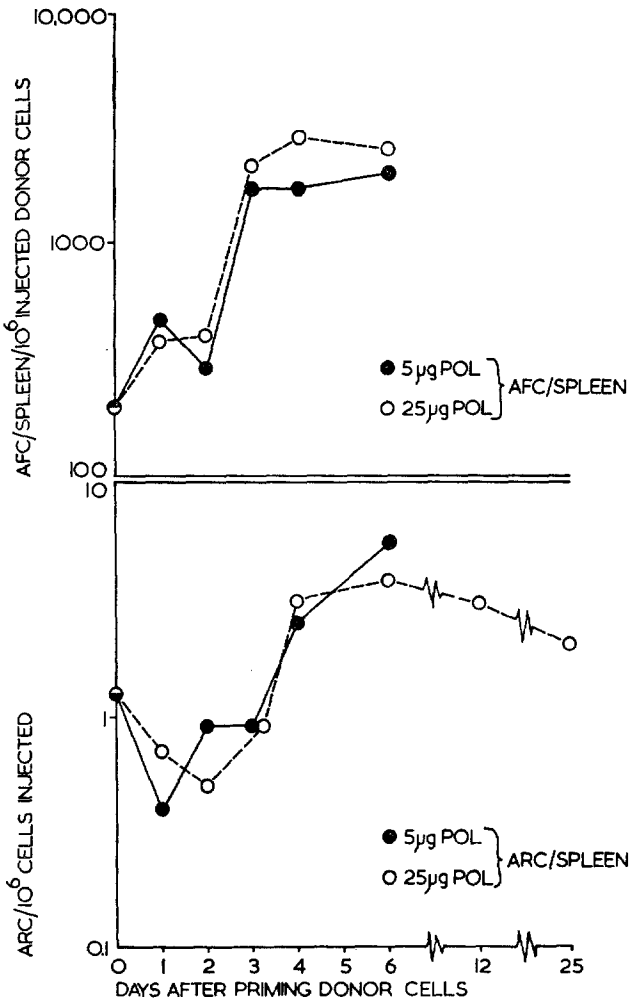

FIG. 2. The AFC and ARC response 8 days after transfer of primed cells to lethally irradiated CBA_{T6T6} mice, and 7 days postantigen challenge. Each point represents the mean response of from 7–19 animals. Using the nonparametric rank test, the depression of 5 μg–1 cells (see text for explanation) and 25 μg–2 cells is significant $P = <0.01$. These depressed responses as well as the response of unprimed cells are also significantly different from 25 μg–4 and 5 μg–4 responses $P = <0.01$.

ARC response was depleted in spleen cell populations which had been primed 1–2 days before transfer (Fig. 2). This decline was greatest for the 5 μg–1 cells and 25 μg–2 cells and statistically significantly different ($P < 0.01$) from the response of unprimed cells. Thereafter, the numbers of ARC/10^6 injected

spleen cells increased, reaching the peak of 5.5 ARC/10^6–injected 5 μg–6 cells, which is not significantly different from 3.7 ARC/10^6 25 μg–6 cells. This peak level which was about three to five times greater than the numbers of ARC found in an unprimed spleen cell population, is statistically significantly different ($P < 0.01$) from the level of ARC in unprimed cells.

Using 25 μg–12 and 25 μg–25 cells, the levels of ARC dropped to 3.1 and 2 ARC/10^6–injected cells respectively. In one experiment involving 30 animals, 2.5 ARC/10^6-injected 10 μg–80 cells were detected, indicating that a plateau of response was maintained from the 25th to the 80th day at least.

These results show that there is an initial depletion of ARC in the primed cell population, which is followed by a rapid augmentation of ARC during the first 6 days of the primary response. The level of ARC drops from five to two times that of unprimed spleen cell populations by 12 days postpriming, and appears to remain at this level for at least 80 days.

Kinetics of AFC during the primary response: The number of AFC detected in host spleens after injection of 25 μg–1 cells was about twice that found in unprimed spleen cells used as controls, i.e., a mean of 390 AFC/10^6 of 25 μg–1 cells versus an average of 160 AFC/10^6 unprimed spleen cells (Fig. 2). The number of AFC in host animals did not rise when 25 μg–2 cells were injected but, when 25 μg–3 cells were used, the number rose to a mean of about 2,200 AFC/10^6 injected spleen cells and a comparable level persisted when 25 μg–6 cells were used. It should be remembered that only about 8% of the primed donor spleen cells remain in the host spleen. Therefore, if this seeding rate is taken into account a more realistic figure would be 2.7×10^4 AFC/10^6 25 μg–3 cells seeded in the host spleen.

No major differences could be detected in the AFC or ARC responses between 5 and 25 μg concentrations of antigen. However, the decline in the ARC response of the cell populations primed with 5 μg of POL and the recovery of this response to the levels of unprimed cells was, in each case, 1 day ahead of those cells primed with 25 μg of antigen. Also, excluding day 1, all 5 μg POL primed donor cells produced consistently lower mean values of AFC/10^6 injected cells after 8 days in the irradiated host.

The ratio of AFC/ARC/10^6 donor cells injected was calculated (Fig. 3) and found to be very high when 1–3 day primed donor spleen cells were employed, e.g., 2467 AFC/ARC for 25 μg–3 cells. When 4-day primed cells were tested, the ratio dropped by a factor of 7, and by day 6 this number was 360 AFC/ARC or only twice that found using unprimed donor cells.

Serum antibody titer: It has been shown previously that an immobilization titer of 1:640 or greater of serum antibody can produce detectable foci in unimmunized irradiated host spleens (6). For this reason, sera from animals receiving primed donor cells were titrated for serum antibody. In one animal the serum titer reached 1:320, and all others were less than 1:160, which is well

below the critical level of 1:640. It is therefore concluded that antibody containing foci detected in the spleens of the host mice in these experiments were not due to circulating serum antibody levels, but rather to antibody derived from foci of AFC cells localized in the spleen.

The ARC Focus Response in Tolerant Mice.—A state of immunological tolerance may be produced in C_{57}BL/Brad mice by using the cyanogen bromide digest of POL (digest). If a dose of 10 μg of digest is given intraperitoneally 6 days/wk beginning at birth, a state of immunological tolerance is usually produced within 6 wk.

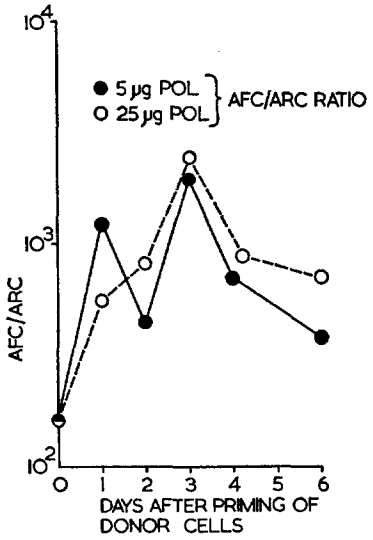

FIG. 3. The ratio of AFC/ARC in primed populations of cells 8 days after transfer into lethally irradiated host mice and 7 days postantigen challenge.

Animals made tolerant in this fashion were tested for the presence of ARC in the spleen before and after various challenges with antigen. Since the digest preparation is normally nonimmunogenic, the monomer of *S. adelaide* flagellin (MON) was used as the challenge antigen, since its physical properties are closer to those of the digest than is POL.

ARC response in normal mice to MON: Compared with results obtained using POL as antigen (6), the response of normal C_{57}BL/Brad spleen cells challenged with 75 μg MON in the transfer system, was 0.5 ARC foci/10 $\times$ 10^6 injected spleen cells or a 4-fold reduction (Tables II and IV). Such a decrease was also observed in the over-all response to MON, since the SA was 5%/10 $\times$ 10^6 donor spleen cells or 14% of that found when POL was used as antigen.

Although the response to MON in untreated mice was considerably reduced

in terms of SA and ARC/10 $\times$ 10^6 injected spleen cells, when compared with that to POL, it was readily detected and therefore used for comparison with the response found with donor cells from tolerant mice.

ARC in tolerant mice: Mice made tolerant by regular intraperitoneal injections of digest were divided into three main groups: (*a*) those which had received no challenge of MON, but had immobilization titers less than 1:5 and were killed immediately after the cessation of digest administration, (*b*) those mice which were killed 1 wk after a single challenge of 75 μg MON, (*c*) those animals killed 1 wk after challenges of 10 and 75 μg MON, which were separated

TABLE IV

Mean ARC in C_{57}BL/Brad Mice after Tolerance Induction with Digest Antigen

Treatment group	Donor cells injected/host mouse	Mean per cent specific activity	Mean ARC foci/spleen	Mean ARC/ 10×10^6 cells injected
A. Normal	8.5×10^6	2.5	0.4	0.5
Normal	9.7×10^6	5.0	0.6	0.6
B. Digest (10 μg) no challenge, tested immediately	19.6×10^6	0	0	0
C. Digest (10 μg) 75 μg MON* challenge, tested 1 wk later	13.7×10^6	0	0	0
D. Digest (10 μg) 10 μg MON*, 75 μg MON 1 wk later, tested 3 wk later	17×10^6	2.5	0.4	0.2
	17×10^6	4.0	1.9	1.1

* The first challenge was given 1 day after the last injection of digest. From 6–22 animals were used in each treatment group.

Using the nonparametric rank test, group A is significantly different from group B at the 5% level. ($P = <0.05$).

by 1 wk. In the latter two groups, all mice used had serum titers of less than 1:5 before the initial challenge.

When antigen injections were maintained up to the time of assay, the spleen cells of the test animal contained no ARC, even when the cell dose was double that normally given. When cell populations were challenged with 75 μg MON 1 wk previous to testing and received no antigen after that challenge, again no ARC were found.

However, mice which received no digest after being challenged with 10 μg and then 75 μg MON 1 wk later, and were tested 1 wk after the last challenge, showed a slight increase in the number of ARC in one group, and a near normal response in another.

Thus, ARC of splenic origin are depleted in C_{57}BL/Brad mice made tolerant by daily digest injections. By challenging the lymphoid system with MON, immunological tolerance appears to give way to a near normal or slightly augmented immune response within 3–4 wk.

DISCUSSION

The main purpose of this study is to obtain information concerning the antigen-reactive cell in unprimed, primed, and tolerant lymphoid cells of the mouse. Of the unprimed cells tested, the presence of ARC in Peyer's patches in relatively larger numbers than the spleen, constitutes a finding of some interest in view of the reports by Cooper et al. (19) and Cooper and Turner (20). In hypothesizing that Peyer's patches are the mammalian equivalent of the avian bursa of Fabricius, Cooper et al. (19) used irradiation and surgical depletion of the relevant tissue in rabbits to show a depletion in the over-all response.

However, the presence of AFC to SRBC and the high level serum antibodies to *Salmonella enteritidis* "H" antigens after direct injections into the Peyer's patches of rats (20) argues against this view, since bursal cells of themselves are not able to produce immunity in an adoptive transfer system (21) nor does the bursa contain plaque-forming cells when immunized against SRBC (22). Our findings of enriched numbers of ARC to POL in Peyer's patches indicate that these tissues not only possess immunocompetent cells, but also contain about twice as many as in spleen cell populations calculated on a per million cell basis and assuming that transferred lymphoid cells seed into the spleen at a constant rate. Thus, removal of this tissue could conceivably reduce the immune response simply by depleting the numbers of readily available ARC.

Recent efforts to obtain an immune response with bone marrow injected with SRBC into lethally irradiated syngeneic mice have been unsuccessful unless thymus cells or TDL are injected as well (4, 23, 24). Our results with POL indicate that bone marrow contains about 30% as many ARC/10^6 cells as does the spleen. This surprising response is further shown to be thymus cell independent since the responsiveness of cells injected into lethally irradiated hosts is not enhanced when large numbers of thymus cells are injected together with the bone marrow cells.

This apparent dissociation of the bone marrow ARC response from thymus cells has been shown to be present in aged, adult thymectomized mice, contrary to findings using the SRBC antigen (25). In unpublished observations by one of us (E. D.) 600-day-old adult C3H mice, thymectomized at 40 days of age, produced serum antibody levels to a POL challenge equivalent to nonthymectomized mice tested of the same age. Humphrey et al. (12) have shown that serum antibody levels in neonatally thymectomized mice are reduced using SRBC and *Salmonella typhi* H antigens in the presence of normal serum levels of IgG, while responses to other antigens such as hemocyanin remain relatively normal. Further, in work done by Martin and Miller[2] serum immobilization titers in neonatally thymectomized CBA mice injected with POL were not reduced when compared with sham thymectomized controls.

[2] Martin, J., and J. F. A. P. Miller. Influence of thymectomy and antilymphocyte globulin, on antibody response to *Salmonella adelaide* flagellin in mice. In preparation.

Thus, a considerable amount of evidence is accumulating which indicates that thymus cells are not required for the maturation of some immunocompetent cells or for the processing of antigen when POL is used. Since there is no doubt that, using the SRBC antigen, the thymus is required in mice for the maturation of AFC from bone marrow precursor cells, we are forced to the conclusion that different antigens such as erythrocytes and POL are processed for the immune response by a different system of cell interaction with antigen. A relatively simple explanation for the difference in handling of the POL and SRBC antigens may be that the size and complexity of the erythrocyte antigen requires intermediate processing procedures by cells such as those derived from the thymus, whereas POL may react directly with the ARC to produce a response.

The ARC response of TDL to POL is 50% of that found in equivalent numbers of spleen cells and considerably less than the response of these cells to SRBC antigens as reported by Miller and Mitchell (5). It has also been reported that the small lymphocyte fraction prepared by passing TDL through columns of glass beads produced identical results with untreated TDL in the SRBC system (14). Our results which show a marked diminution of the ARC response in column-refined cells to less than 30% of the response of ARC in untreated TDL, coincide with the results of Nossal et al. (14) who reported a similar situation, but using serum titer as an index. As these authors point out, this difference may be considered due to a secondary response to the SRBC antigen as a result of partial priming by cross-reacting antigens present in enteric bacteria (26) which may enter the blood in small amounts from the gut. Another possible explanation for the diminished response to column refined TDL is that ARC to POL are not small lymphocytes as is believed to be the case with the SRBC antigen (27).

It is of further interest to note that when TDL and bone marrow cells were injected together into irradiated hosts, the number of responsive ARC did not increase, but there was a 30% increase in SA when compared with the additive total SA of both cell population responses tested separately. This suggests that the response of the ARC may be enhanced regarding antibody production within each ARC focus rather than by increased numbers of ARC. There are three possible causes of this enhancement: (*a*) increased production rate of antibody by the individual cell, (*b*) more avid antibody, or (*c*) the presence of more AFC/ARC focus. Recent evidence by Mitchell and Miller (24) lends support to the latter possibility, as they were able to show increased numbers of AFC/ARC in mice injected with bone marrow, TDL and SRBC, but no increase in the number of ARC as a result of this combination of cell populations.

Further studies would be needed to show that this is the reason for the increased SA using POL in the bone marrow–TDL combination, and if it was, whether the increased number of AFC/ARC was due to increased mitotic rates or other factors.

Sercarz and Byers (15) reported that recall of IgM antibody production in the mouse to SRBC antigens can be detected by day 1 after the initial dose of antigen. These experiments were done in whole animals and the memory response measured in terms of plaque-forming cells (PFC) and the "net excess secondary response" (16). The authors were unable to quantitate the response at the level of the ARC, and therefore their observations may be interpreted as resulting from more efficient antigen trapping because of the presence of specific antibody, or possibly due to the further stimulation of already committed and dividing AFC cells. This latter possibility assumes that the AFC could recognize the presence of more antigen and be driven to further divisions, which has been shown to occur in serial transfer experiments by Möller (28).

The adoptive transfer system described in this paper permits the investigation of the numbers of ARC in a given primed cell population as well as the numbers of AFC. The main limitations of this system are that ARC and AFC responses can only be quantitated and that the architecture, and consequently spatial relationships, of the primed donor cells are disturbed at the time of transfer into the lethally irradiated host. Since the host animal is lethally irradiated, only those cells which were transferred will be able to proliferate and problems of recruitment from bone marrow or other lymphoid tissues do not have to be considered.

Our results indicate that the numbers of responding ARC dropped for the first 2 days after priming, rose to the levels of unprimed cells by the 3rd day, and by the 6th day were four to five times greater than the levels obtained from umprimed cells. The primed donor cell population of AFC rose steadily to a peak at day 3 and thereafter plateaued till day 6. However, the question arises, whether or not the cells which populate the foci detected in this assay are the direct progeny of an ARC stimulated in the host or the result of continued proliferation of dividing transferred AFC. The fact that the ARC level remained low or dropped while the AFC response rose, and also that the peak response of ARC was 1–3 days later than the AFC peak, suggests that dividing AFC probably do not produce foci. Perhaps the most reasonable interpretation of these results is that the initial depletion and subsequent augmentation of ARC may be attributed to a cell line independent of the injected AFC.

The high ratios of AFC/ARC present in the host spleen containing donor cells, primed at various intervals before transfer, pose an interesting problem. Do the results indicate that there are more AFC progeny from a single ARC or have the transferred AFC continued to divide in response to the second challenge of POL? The increase in numbers of AFC is 5–10 times greater than AFC/ARC levels found in unstimulated spleen cells and could be explained most easily if one assumes that the injected AFC continued to divide about 4–5 times after transfer, assuming that about 8% of the AFC settle in the spleen. Möller (28)

has shown that the division of primed cells serially transferred to irradiated syngeneic mice is able to continue on for about 38 divisions in the presence of fresh antigen, thus giving some support to our proposal. Further, by the 6th day the ratio of AFC/ARC has dropped to about three times the level of unprimed cells, possibly due, in part, to the increased number of responding ARC, feedback mechanisms (29), and cell migration from the spleen (30). However, these observations do not rule out the possibility that the ARC responding in this system are able to produce larger numbers of AFC than are ARC from unprimed populations.

To the present time, information on the primary response has been obtained primarily with serum antibody levels and the assay of single AFC. The ARC focus assay has permitted the broadening of this information to the enumeration of the ARC, but it has not permitted the qualitative differentiation between ARC and "memory cells." However, the ultimate rise in numbers of ARC 4 days postantigen, which remains elevated for at least 80 days, is probably a result of memory production in the primary response.

To date, data on tolerance in mice have been derived from serum antibody studies, except for recent studies at the cellular level in mice made tolerant to SRBC using cyclophosphamide (31). The use of flagellin of *S. adelaide* to produce low and high zone tolerance in neonatal Wistar rats has been reported by Nossal et al. (32) and Shellam and Nossal (33). The cyanogen bromide digest of this antigen is able to produce tolerance in adult Wistar rats as described by Parish et al. (8).

Using the latter method in neonatal C_{57}BL/Brad mice for the tolerance experiments described herein, it appears that (*a*) tolerance may be established in C_{57}BL/Brad mice using digest, (*b*) this tolerance is not broken spontaneously within 8 days after transfer to lethally irradiated hosts and 7 days post-MON challenge, confirming similar studies in C_{57}BL/Brad mice (34), (*c*) tolerance may be broken in those animals killed 1 wk after challenges with doses of 10 and 75 μg MON which were separated by 1 wk. The results show that the lesion of tolerance is at the level of the ARC or its precursors, which supports the concept of central failure as put forward by several investigators (see review by Dresser and Mitchison, reference 35). This is also supported by in vitro studies (36). Cells made tolerant in vitro to POL, when transferred to an irradiated host and subsequently challenged, retain their tolerant state for at least 8 days (37).

It is hoped that further studies in vivo and in vitro will elucidate the kinetics of tolerance at the level of the ARC.

SUMMARY

The numbers of antigen-reactive cells (ARC) responding to a purified protein, the polymer of *S. adelaide* flagellin, have been assayed in cell populations derived from several lymphoid tissues of mice. The assay, which employs the cell trans-

fer into lethally irradiated mice, indicates that there is a response of ARC in bone marrow in the absence of thymus cells. This suggests that the immune response to this protein antigen is not thymus dependent.

The presence of relatively large numbers of ARC in Peyer's patches argues for their direct participation in the immune response in the adult mouse.

The kinetics of ARC and antibody-forming cells in the early primary response employing the transfer system is described. The numbers of ARC declined during the first 2 days of the immune response, but by day 6 had increased to about five times the number in unprimed spleen cells. The rise is believed to be a result of the primary injection of antigen and therefore may be described as memory; however, these experiments have not been able to further elucidate any specific qualities of the "memory cell."

Tolerance induction in C_{57}BL/Brad mice produced by repeated injections of a cyanogen bromide digest of the antigen is described. The ARC or its precursor is shown to be the site of the lesion of tolerance by direct investigation.

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