A NEW METHOD FOR THE ENUMERATION OF ANTIGEN-REACTIVE CELLS RESPONSIVE TO A PURIFIED PROTEIN ANTIGEN*,‡

By W. D. ARMSTRONG,§ M.D., and E. DIENER, Ph.D.

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

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Although the kinetics of the antibody-forming cell (AFC)[1] in the mammalian immune response has been determined in great measure, much has yet to be learned about the antigen-responsive precursors of these cells. Recently, progenitors of AFC have been enumerated and characterized (1–4) by injecting known numbers of mouse spleen cells and sheep erythrocytes (SRBC) into lethally irradiated syngeneic mice and after 8 days testing the host spleens for foci of hemolysin production. It was reasoned that where foci of hemolysin were detected, progenitors of AFC had proliferated into discrete colonies of hemolysin-producing cells. The predictability and linearity of the cell dose–response curve obtained in such experiments supports the belief that each of these foci resulted from the proliferation of one antigen-stimulated immunocompetent donor cell, or what we prefer to call the antigen-reactive cell (ARC) for reasons previously stated (5).

The purpose of this paper is to describe a technique which permits the enumeration of the ARC in various parts of the mouse lymphoid system, using the polymerized flagellin of *Salmonella adelaide* as antigen. The method is modeled after that of Kennedy et al. (1) and utilizes the principle of immobilization of flagellated *Salmonella derby* bacteria by the relevant anti-flagellar antibody as described by Nossal (6).

The main advantage of this technique lies in the fact that it permits the enumeration of ARC in brisk and well-studied primary and secondary responses, as

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§ Supported by a Medical Research Council of Canada Fellowship. Present address: Department of Pathology, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada.

[1] The following abbreviations are used in this paper: AFC, antibody-forming cell; SRBC, sheep erythrocytes, ARC, antigen-reactive cell; and POL, polymer antigen of *S. adelaide* flagellin.

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well as in the important phenomenon of immunological tolerance. By employing pure protein antigens, such as the polymer of *S. adelaide* flagellin, immune responses may be investigated using either the soluble or particulate forms which can be readily labeled with radioactive isotopes. These qualities allow us not only to investigate the parameters of the ARC in another antigen system different from that of the complex erythrocyte antigen, but also to explore in greater breadth more facets of the immune response.

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, and powdered milk, and postirradiation were housed in groups of five or less per cage. Early experiments done on mice of both sexes indicated there were no significant sex differences in the immune response; however almost all experiments thereafter used male mice. Hall Institute mice of about 15 wk of age were used for the production of hyperimmune serum. Prior to all intravenous injections mice were kept at 37°C for 15 min to produce dilatation of the tail veins.

Irradiation.—Mice were irradiated using a Philips (R.T. 250) 250 kev machine at 15 ma and 0.8 mm Cu HVL. Doses between 400 and 1000 rads were applied in these experiments, and in some cases a split dose schedule with an interval of 7 days was employed to reduce the short-term irradiation mortality in CBA_{T6T6} (400 + 600 rads) and $C_{57}BL/Brad$ mice (325 + 425 rads).

Antigen.—Polymer antigen (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 antigen were made in saline except that used for the production of hyperimmune serum, where 10 μg of antigen was mixed in complete Freund's adjuvant and injected intraperitoneally into each mouse. This dose of antigen with adjuvant was reinjected intraperitoneally 30 days later, and the animals were bled 2–4 wk later.

Serum Titrations.—All sera were separated from the blood clot within 24 hr and frozen for storage. Doubling dilutions of serum were titrated using the bacterial immobilization technique (8).

Bacteria.—The bacterial strain used as indicator in this study was *S. 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 (0.6% gelatine and 0.4% shredded agar in heart infusion broth). A large enough cube of agar and bacteria from the stock plate was placed in bacterial nutrient broth, so that by 2 hr of incubation at 37°C, the optical density of the solution at wavelength 600 mμ was 78% transmittance,[2] or approximately 5×10^7 organisms/ml.

Preparation of Bacterial Slides.—Of the standardized bacterial solution, 2 ml were added to 18 ml of molten nutrient gelatine agar (0.4% agar, and 7% Davis gelatine in heart infusion broth, which was adjusted to pH 7.3 using 1/N NaOH) mixed and kept at 42°C in a 21 ml Perspex container designed for the dipping of microscope slides. Within 15 min, clean microscope slides were dipped in the solution of bacteria, allowed to drain momentarily, and the undersurface of the slide wiped clean, leaving a thin layer of agar and bacteria. The bacterial concentration on the upper surface of the slide was then approximately 2×10^6 bacteria/mm^2 of slide surface.

Once coated, the slides were kept in standard plastic Petri dishes (diameter, 9 cm) which contained a wet filter paper and two wooden dowels each 2 cm long, for the purpose of keeping

[2] Using a Bausch and Lomb, Spectronic 340.

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the slide off the wet filter paper. It should be noted that the filter paper must be moist enough to keep the slide from drying, yet with no excess water which might dilute the agar on the slide by capillary action. Since bacterial activity on the slide decreased with time, slides could not be stored overnight and were usually used within 3 hr of dipping.

Cells.—Using aseptic technique, the mouse spleens were minced with scissors, and gently forced through a stainless steel sieve into a solution of 30% fetal calf serum in Eisen's balanced

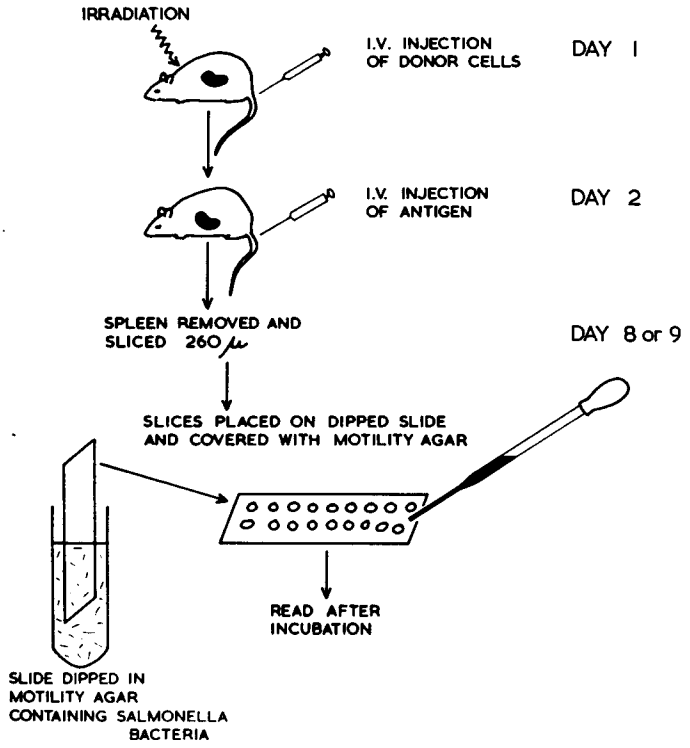

FIG. 1. Schematic representation of the assay for antigen-reactive cells using the polymer antigen of *S. adelaide* flagellin.

salt solution (9). The suspension was placed in a 5 ml test tube, allowed to stand for 5 min at 4°C and the supernatant removed, leaving behind the sedimented debris. The cells were counted using a hemocytometer, diluted to the appropriate concentration, and a check count performed before injection into the mouse tail vein.

Assay Method for ARC.—The technique is summarized in Fig. 1. Syngeneic donor cells were injected into the host mice about 4 hr after irradiation, and antigen was injected 24 hr later; all injections were done via the lateral tail vein.

On the appropriate day after irradiation (usually day 8) animals to be bled were anesthetized and exsanguinated by cardiac puncture, otherwise killed by cervical dislocation, after which the spleen was removed. The spleen was moistened with Eisen's balanced salt solution, placed on a 4 cm square, clear, polyethylene sheet, and clamped to the stage of a modified McIlwain tissue chopper (10) where it was chopped into slices 260 μ thick. The

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slices were placed in order on the slides coated with bacteria and agar and within 15 min were covered by dropping clean nutrient gelatine agar maintained at 42°C onto the slices. The slides were incubated for 2.5–3 hr at 37°C, to allow complete freedom of movement of the bacteria in the agar, kept at room temperature overnight (15–18 hr), and read next morning employing a dissecting microscope at a magnification of 10–20 ×,[3] using reflected light and a black background.

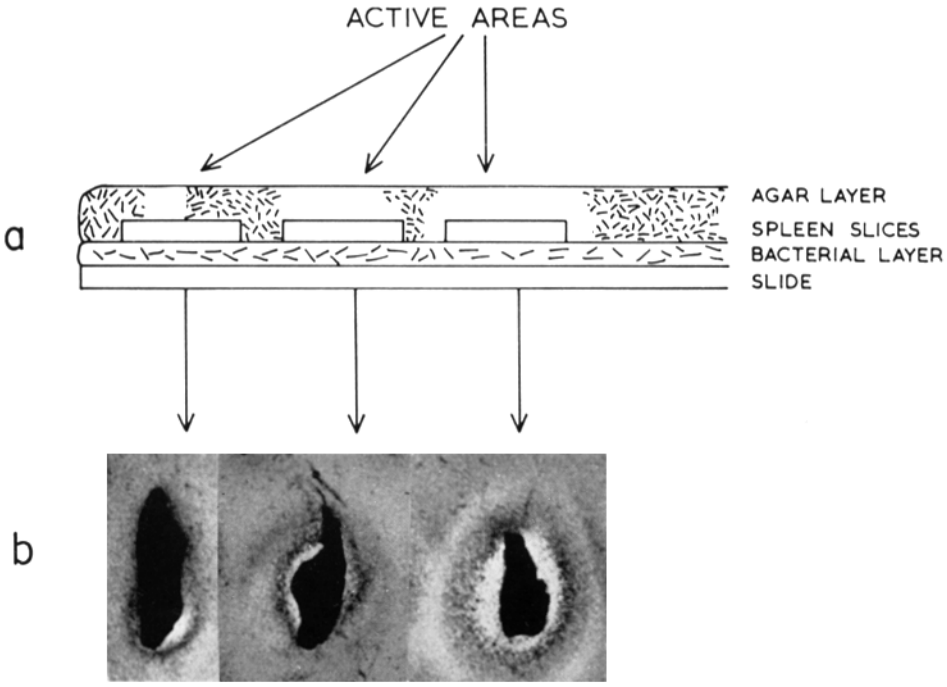

FIG. 2. (*a*) Diagrammatic representation of the composition of a test slide, agar, bacteria, and spleen slices after incubation. (*b*) Photographic appearance of the range in size of the area of inhibited bacterial movement due to the antibody from positive spleen slices. × 10.

The scoring of positive slices shown in Fig. 2 is based on the observation that antibodies to the flagella of *S. adelaide* will immobilize but in no way affect the viability of the indicator bacteria, *S. derby*, in the agar around the spleen slices. Where antibody is present in large enough amounts to diffuse into the agar, the bacteria are immobilized and unable to swarm over the area. However, the rest of the surface is covered with an even, opaque growth of bacteria, thus giving a readily visible contrast between positive and negative. A focus may be defined as one or more contiguous positive slices separated from other positive areas by one or more negative slices.

The number and size based on the total number of positive slices of each focus were recorded as was the total number of slices for each spleen. When all the slices of a test spleen were negative, the results were included in the calculations, but when all the slices of a test

[3] Stereo microscope, Wild M.5, Heerbrugg, Switzerland.

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spleen were positive (confluence), the results were not included. All of the statistical limits quoted in the data are 95% confidence levels as calculated by doubling the standard error of the mean.

AFC Assay.—The AFC were enumerated using the adherence colony technique described by Diener (11). This detects antibody-forming cells by the method of bacterial immunocytoadherence, a sensitive and specific assay in this system.

Trapping Efficiency of Injected Donor Cells by Host Spleens.—An experiment was done to determine the percentage of transferred donor cells responding to 25 μg of antigen which was injected into CBA_{T6T6} mice 24 hr after lethal irradiation and injection of the donor cells.

TABLE I

Background Foci per Spleen in the Total Number of Control Mice Sacrificed Days 8 and 9 Postirradiation

Mouse strain	Irradiation dosage	Antigen dosage	No. cells injected	Background foci/spleen
	rads	*μg*		
CBA_{T6T6}	800	25	—	14/219—0.07 ± 0.04*
	1000 SD	25	—	2/30 —0.07 ± 0.06
	800	—	$1–2 \times 10^6$	0/95 —0
	800	—	5×10^6	0/15 —0
	800	—	10×10^6	0/6 —0
	—‡	—	—	0/10 —0
$C_{57}BL/Brad$	650	25	—	2/43 —0.05 ± 0.04
	750 SD	25	—	0/30 —0
	750 SD	—	2×10^6	0/5 —0

SD, split-dose irradiation; CBA_{T6T6}, 400 rads plus 600 rads; $C_{57}BL/Brad$, 325 rads plus 425 rads.

* Indicates 95% confidence limits.

‡ Normal animals receiving no treatment and tested for the possibility of preexisting foci of antibody.

In order to establish the focus-producing potential of the test cell suspension, each of 10 host animals prepared in the usual fashion was injected with 2×10^6 cells, given 25 μg of antigen 24 hr later, and assayed after 7 days. At the same time, 50×10^6 of the test cells were injected into several other irradiated hosts which were sacrificed 24 hr later. The spleens of these animals were pooled, cell suspensions made, and 50×10^6 cells were injected into freshly prepared hosts. These animals were given 25 μg of POL the following day, sacrificed 7 days later, and their spleens assayed for ARC foci. Using calculations described by Kennedy et al. (12), this experiment indicated that 5% (±2%) of injected donor cells remained within the spleens of four surviving CBA_{T6T6} mice and were responsive to the antigen given 24 hr later.

RESULTS

Background.—Several different controls were established for both strains of mice to determine how many and under what conditions background foci occurred.

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1. Initially all experiments included two major controls: (*a*) host mice which received varying numbers of donor spleen cells within 4 hr of irradiation and saline instead of antigen the next day; (*b*) host mice which received only the medium used for cell suspensions after irradiation plus 0.5–50 μg of antigen 24 hr later. A summary of these results is presented in Table I which includes the

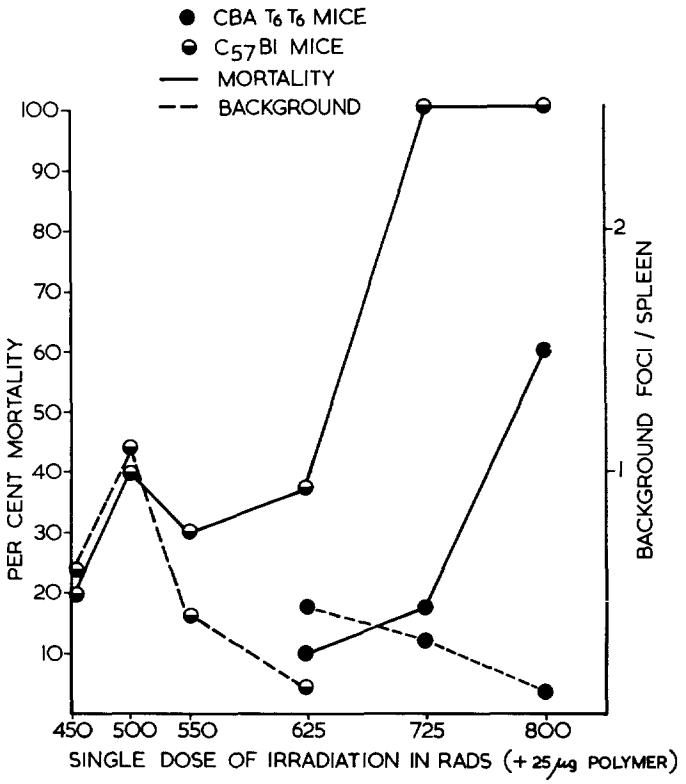

FIG. 3. The effect of increasing amounts of irradiation on the background present in CBA_{T6T6} and $C_{57}BL/Brad$ mice. All animals received 25 μg of polymer antigen 24 hr after irradiation and all spleens were assayed 8 days postantigen. Each point represents the mean results of 6–10 animals.

results of both CBA_{T6T6} and $C_{57}BL/Brad$ mice and both types of irradiation used. No background foci were detected in those animals receiving cells only, and a mean of 0.07 ± 0.04 foci per spleen was detected in those animals receiving antigen only. In the case of the $C_{57}BL/Brad$ mice, the split dose of irradiation reduced the background focus level to zero and decreased the over-all mortality before sacrifice.

2. Since background foci were present in irradiated animals receiving antigen

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only, it was reasoned that the background could be related to incomplete destruction of the immune potential by irradiation. Therefore, an experiment was performed in which mice received varying amounts of irradiation and 25 μg of antigen 24 hr later. As the amount of irradiation increased to the 100% lethal dose by 8 postantigen days, the background level dropped to about 0.1 focus per spleen (Fig. 3), and any attempt to remove background entirely by increasing the irradiation dose produced unacceptably high mortality rates. This suggests that the background which we experience is produced by cells not destroyed by the amounts of irradiation used in these experiments. Furthermore, we have shown that although the C_{57}BL/Brad mice have a lower LD_{50} resulting from irradiation, the disappearance of background also occurred at a lower irradiation dose as compared with that of CBA_{T6T6} mice.

3. It has been shown by others that the background response to erythrocyte antigens injected 24 hr after the animals are lethally irradiated is much reduced when compared with the response detected if the antigen is injected immediately after irradiation (13). This observation has been tested with the polymer antigen of *S. adelaide* flagellin by injecting CBA_{T6T6} and C_{57}BL/Brad mice with 25 μg of antigen together with donor spleen cells (combined injection) within 4 hr of irradiation in the first series, and the same concentration of antigen 24 hr after irradiation and cells in the second series (split injection). A control series of animals was divided into two groups to test for background; the first group was injected with antigen only within 4 hr of irradiation, and the second group received antigen 24 hr after irradiation. When tested for foci 7–8 days later, both strains of mice had reduced background levels using the split injection, the CBA_{T6T6} by 1 focus/spleen (83%) and the C_{57}BL/Brad by 0.2 foci/spleen (100%) (Fig. 4). At the same time, the number of foci measured in the series of test animals did not decrease with the background after the split injection, thus giving a relative increase in the number of foci. In spite of the possibility of increased mortality due to the 24 hr delay using the split injection technique, this schedule was adopted because of the lower background level of foci and the higher production of foci per 1×10^6 cells injected. Further, this experiment confirms the observations of others (13) and also suggests that the background in our test system is probably related to the incomplete destruction of the immune potential of the host following lethal irradiation.

4. Over 120 animals received cell injections varying from 1×10^6 to 10×10^6 cells after lethal irradiation, but only saline, rather than antigen, was injected 24 hr later. Background foci were not detected in a single spleen in these control mice (Table I), and since no antigen was injected, this also supports the belief that background foci are a result of the stimulation by antigen of host ARC which have escaped irradiation.

Relationship of ARC Foci to the Presence of Specific Antibody.—To demonstrate that foci were in fact due to specific antibody, spleens from CBA_{T6T6} mice

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which were previously injected with 10×10^6 donor spleen cells (to ensure that all slices were positive) and 25 μg of antigen 24 hr later, were removed, sliced, and placed on slides coated with the bacteria in agar mixture. Enough undiluted

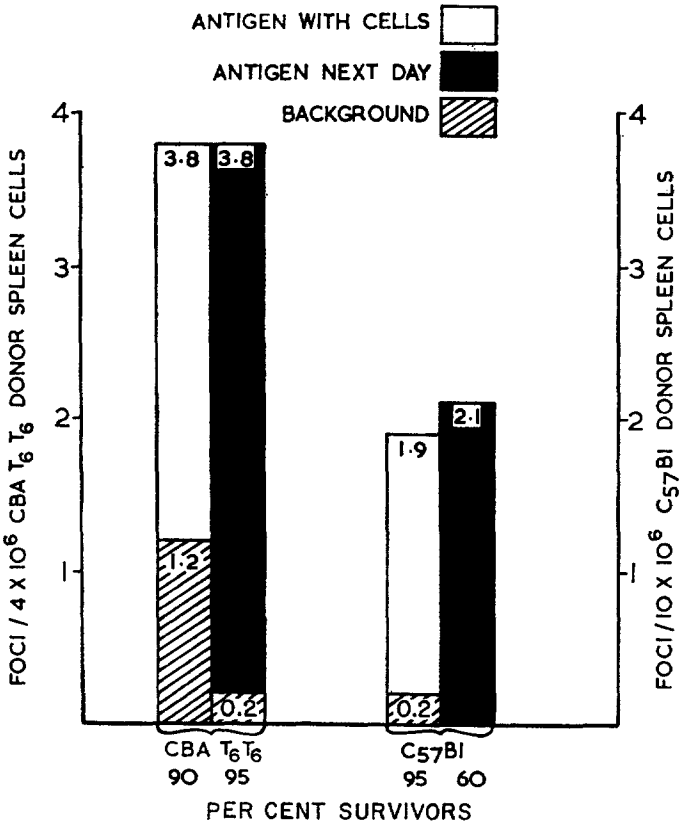

FIG. 4. The effect of delayed antigen injection on irradiated and spleen cell grafted CBA_{T6T6} and $C_{57}BL$/Brad mice. Each strain of mice was divided into two main groups after irradiation; one group was injected with cells and 25 μg of antigen within 4 hr of irradiation—plus a control group of animals receiving antigen only at the same time. The second major group was injected with cells only within 4 hr of irradiation and the following day given antigen. Controls had not been given cells after irradiation, but did receive antigen 24 hr later. All spleens were assayed 8 days after antigen, and the percentage of animals in each category surviving at the time of assay is recorded at the foot of the graph. From 5–10 control animals were tested in each group, and each test group was made up of from 10–15 animals.

rabbit anti-mouse gamma globulin to cover a single slice was dropped onto alternate slices, the entire preparation overlayed with clean agar and incubated for the appropriate period of time. The results showed that only those slices which were not covered with anti-mouse gamma globulin were positive, indi-

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cating that ARC foci are antibody dependent. Conversely, mouse serum hyperimmune to the polymer of *S. adelaide* flagellin was dropped onto a slide coated with bacteria and agar, then covered with clean agar, incubated, and where each drop was laid a simulated positive area was produced. In order to determine the amount of antibody needed to simulate a focus, purified rat IgM and IgG were serially diluted with molten nutrient gelatine agar and 2 μl drops/dilution were placed in order (and well separated) on a slide coated with bacteria in agar; this was covered with clean agar and then incubated for the appropriate time and temperature. About 3 ng of IgM and 5 ng of IgG produced detectable positive areas thus demonstrating that (*a*) foci may be produced by purified antibody in

TABLE II

The Effect of Serum Titer on Focus Formation in CBA$_{T6T6}$ Mice

Serum titer	Spleens with no foci	Spleens with foci
Zero—<1:2*	18	0
1:80 -1:320	7	0
1:640–1:2560	2	7
1:2560+	0	8‡

* No hyperimmune serum given.

‡ All spleens demonstrated "confluence."

All mice were irradiated with 800 rads and injected with 4–5 $\times$ 10^6 normal spleen cells, but given no antigen. Varying amounts of hyperimmune serum were injected intravenously 15 hr before sacrifice in those mice demonstrating a serum titer.

relatively small quantities and (*b*) that the method is approximately as sensitive as the immobilization titration technique used on serum samples (14).

Since very low levels of antibody may be detected in this assay system, it was considered necessary to determine whether serum antibody in test animals could possibly mimic ARC foci in the spleen. Normal CBA$_{T6T6}$ mice were irradiated and injected with 4 $\times$ 10^6 nucleated donor spleen cells and, on the 7th post-irradiation day, serum from hyperimmune mice was injected into the host animals in varying amounts. The animals were killed the following day, the blood saved for serum antibody titrations, and the spleens tested for the presence of foci. The results, summarized in Table II, indicate that mice given hyperimmune serum had no detectable foci in their spleens if the immobilization titer was 1:320 or less, whereas those animals with serum titers of 1:640 or greater had one or more foci in the spleen. During the early part of this work, all animals assayed for foci were also tested for serum antibody titer, and it was found that the maximum serum antibody level was 1:512 in two of 240 CBA$_{T6T6}$ mice tested, while the remaining group of mice had serum titers of less than 1:25. The reason for the presence of foci at higher levels of serum antibody is not

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known; nevertheless it is clear that the ARC foci present in spleens of host animals receiving 5×10^6 (or less) donor spleen cells was not a result of serum antibody in the spleen, since serum antibody levels in test mice are too low to produce nonspecific foci.

The Relationship of AFC and Antibody Foci in the Spleen.—Although we have shown that ARC foci are antibody dependent, the question still remains, whether or not AFC are present in the areas where antibody is detected. In

TABLE III

The Correlation of AFC with CBA$_{T6T6}$ Mouse Spleen Slices Which Contain Antibody-Producing Foci

Animal	AFC/negative slice*	AFC/positive slice‡	Total positive slices in each focus	Total AFC/focus
Control				
1	0	11	1	11
2	0.6	—	—	—
Test				
1	2.8	877	4	3510
2	6.1	—	—	—
3	8.6	(*a*) 43§	5	213
		(*b*) 49	4	195
		(*c*) 55	2	110
		(*d*) 29	2	58
		(*e*) 9	2	18

* All negative slices from one spleen were pooled before testing for AFC.

‡ All positive slices from one focus were pooled before testing for AFC.

§ (*a*)–(*e*) represent five separate foci present in the spleen of test animal 3.

All animals were irradiated with 800 rads, the test mice received 2×10^6 normal mouse spleen cells and the controls, medium only. All animals received 25 μg of POL 24 hr later and were tested on the 7th postantigen day.

order to answer this question, the assay system was modified so that the presence of antibody could be detected in a given spleen slice, and the slice then minced finely to prepare a cell suspension which could be assayed for AFC. The host mice were irradiated as usual, given 2×10^6 normal spleen cells followed by 25 μg of POL after 24 hr, and sacrificed 7 days later. Instead of placing the spleen slices on agar, they were immersed sequentially in wells containing 0.2 ml of Eisen's balanced salt solution and 10% fetal calf serum, incubated for 30 min at room temperature, removed, and placed sequentially in wells of fresh medium. The medium in each of the wells in which the slices were initially incubated was assayed separately for the presence of antibody, employing the immobilization technique used for serum assay. All contiguous slices which had released detectable antibody during the incubation were grouped together and a

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cell suspension made by pressing them through a very fine platinum wire mesh with fine curved forceps. The resulting cell suspension was then assayed for the number of AFC, using the adherence colony technique. Initially, all negative

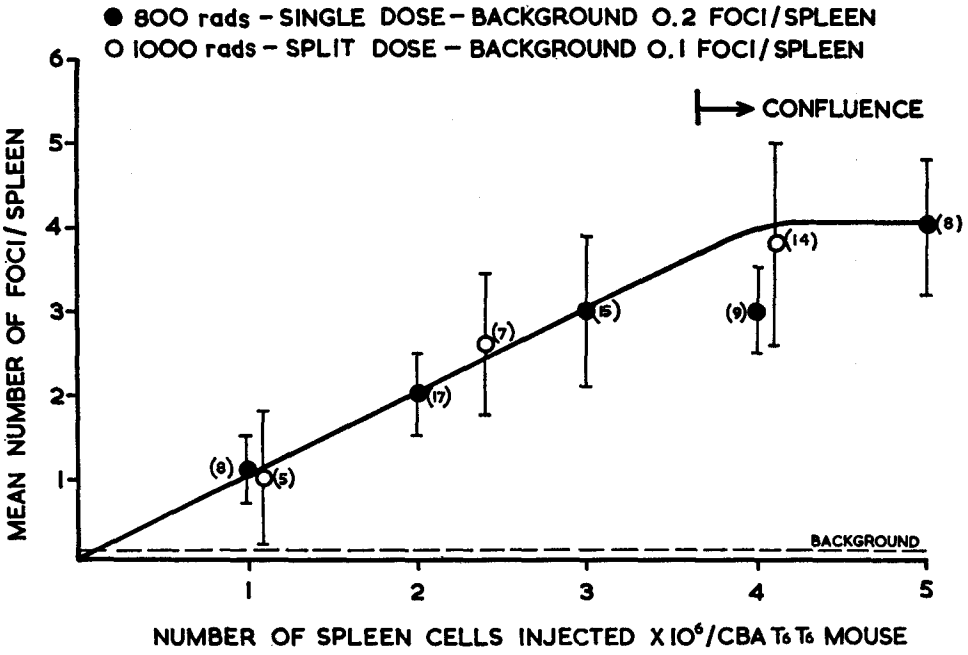

FIG. 5. The ARC focus response in CBA_{T6T6} mouse spleens related to the injected cell concentrations. The bar and arrow mark the point at which "confluence" develops, and hence where the accuracy of reading is reduced. All animals included were tested eight days post-antigen and each point represents the results of at least two experiments done on separate days. The mean background of all the control spleens for this experiment is plotted as the broken horizontal line.

The numbers in brackets represent the number of test animals used to determine that point, and the vertical bars on each point represent the 95% confidence limits. Using the nonparametric rank test, the response to 3×10^6 cells injected is significantly different from 1×10^6 and 2×10^6 cells injected ($P < 0.01$).

slices were combined and treated as one group, the AFC enumerated and expressed in terms of AFC per slice as well as AFC per focus.

The results of this experiment support the belief that the areas of the spleen containing antibody do in fact contain AFC. Several experiments were done, a representative of which is summarized in Table III, wherein all but one area of detectable antibody contained more AFC per slice than a background focus detected in the negative control. The presence of AFC in the negative areas indicates that there is probably some migration of the AFC from the main areas of

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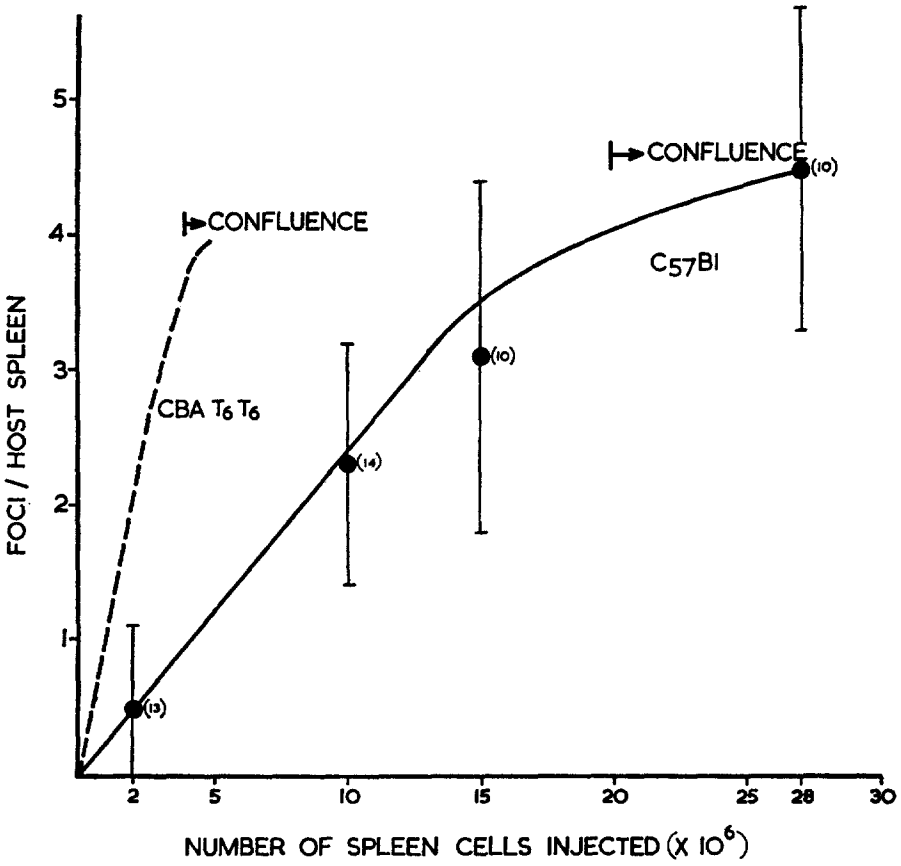

FIG. 6. The ARC focus response in C_{57}BL/Brad mouse spleens related to the injected cell concentration. The broken line represents the curve for the CBA_{T6T6} mouse response drawn in Fig. 3 for comparison with the solid line representing the C_{57}BL/Brad mouse response. The numbers in brackets represent the numbers of animals used to determine each point and are the result of at least two separate experiments. The over-all background of 0.05 (±0.04) foci/spleen has not been subtracted in the calculation of these points.

The vertical bars represent the 95% confidence limits of each point. Using the nonparametric rank test, the response to 10×10^6 cells injected is significantly different from 2×10^6 ($P < 0.01$) and 27×10^6 cells injected ($P < 0.05$).

antibody formation or that some small foci are not detected using this technique. The method is not considered accurate enough to assess actual numbers of AFC per focus because of cell loss and damage due to the 3–4 hr lag from time of sacrifice until the time of plating and the mechanical manipulations the cells must undergo during that period. These factors may also explain why the occasional antibody-producing area contains relatively low numbers of AFC.

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ARC Focus Response Related to the Number of Injected Cells.—The relationship between the number of nucleated donor spleen cells injected into lethally irradiated mice and the foci present in the spleen 7 days after receiving antigen was studied in CBA_{T6T6} and $C_{57}BL/Brad$ mice. This crucial experiment to determine the validity of the procedure indicated that the ARC focus response for CBA_{T6T6} mice is about 1 ARC focus/10^6 nucleated donor spleen cells injected and obeys a straight line relationship over the limited range of 1×10^6 and 3×10^6 cells injected (Fig. 5); this line extrapolates through zero and is equal to a slope of one. If 4×10^6 or more nucleated spleen cells were injected into host mice, many if not all spleen slices were positive, and this "confluence" of positive slices whether complete or almost complete, made it impossible to enumerate the number of ARC foci as accurately as when lower cell concentrations were injected. This phenomenon resulted in lower numbers of detectable ARC foci than expected, producing a plateau effect and a slope much less than one at injected cell concentrations greater than 3×10^6/mouse.

There was no significant difference in the CBA_{T6T6} response when the irradiation dosage was varied between 800 (single dose) and 1000 rads (split dose) or when the assay for ARC foci was carried out on the 7th or 8th postantigen day.

To test the feasibility of this assay system in another strain of mice, $C_{57}BL/Brad$ mice were chosen and, when compared with the CBA_{T6T6} mouse reponse, there was a marked reduction in the number of ARC/1×10^6 $C_{57}BL/Brad$ donor spleen cells (Fig. 6). The curve depicting the ARC focus response between 2×10^6 and 10×10^6 injected nucleated spleen cells again is a straight line extrapolating through zero, and 5×10^6 donor cells are required to give 1 focus/spleen. Some confluence of positive spleen slices occurred when 28×10^6 donor spleen cells had been injected into the irradiated hosts, producing a similar plateau effect as in the response of CBA_{T6T6} mice.

ARC Focus Response Related to Antigen Concentration.—Using the focus assay for ARC and the adherence colony assay for AFC, the effect of antigen dose on immunologically responsive cells may be answered. Concentrations of antigen ranging from 0.5–50 μg were injected into CBA_{T6T6} mice which had been irradiated and received 2×10^6 nucleated donor spleen cells 24 hr previously. The mice were divided into two groups and sacrificed 7 and 8 days after antigen injection. Spleens from the first group were assayed for the number of ARC and those of the second group for the number of AFC.

The results of this experiment (Fig. 7) indicate that by increasing the dose of antigen from 0.5 to 10 μg, significantly more ARC foci are produced per spleen. However, higher doses of antigen do not increase this number further. Spleens were assessed for AFC at the various concentrations of antigen, and there was a statistically significant increase in numbers between the 0.5 and 10 μg dose range, but a drop with a dose of 25 μg. When the numbers of AFC/ARC focus are calculated, there is a decrease from an average 680 ± 380 AFC/ARC at 0.5

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μg to an average 160 $\pm$ 100 AFC/ARC at 25 μg, a difference which appears significant using the 95% confidence levels as error limits to calculate the cumulative proportional error. Therefore, between the concentrations of 0.5 and 10

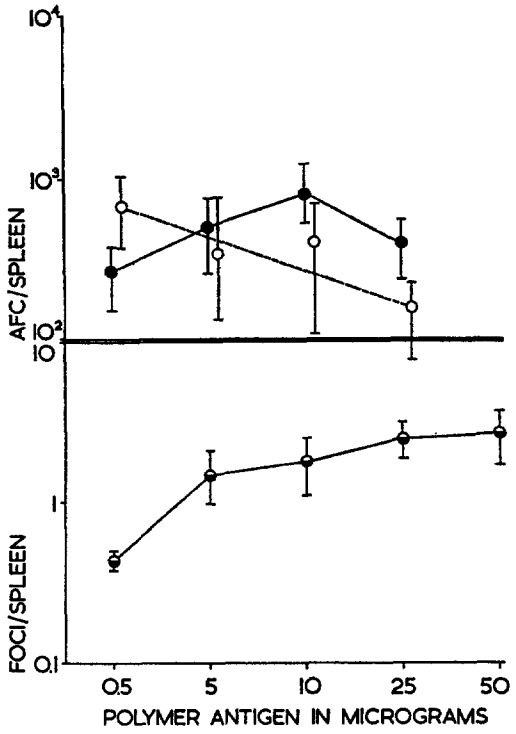

FIG. 7. The ARC focus and AFC response in CBA_{T6T6} mouse spleens related to the concentration of injected antigen. All animals received 800 rads and 2×10^6 spleen cells 24 hr before antigen was injected and were tested 7 and 8 days postantigen.

◒, arithmetic mean response of ARC foci per spleen; ●, arithmetic mean response of AFC per spleen. The vertical bars on these points represent the 95% confidence limits. Each of the above points represents the arithmetic mean value of 15–25 animals. ○, the ratio of AFC/ARC. The vertical bars indicate the cumulative proportional error, calculated by using the 95% confidence intervals as error limits. The background of 0.05 $\pm$ 0.04 foci per spleen has not been subtracted from the ARC focus response, but all AFC values are corrected for background. Using the nonparametric rank test, the responses of both AFC and ARC at the antigen dose of 10 μg are significantly different from the responses using 0.5 μg of antigen. ARC, $P < 0.01$; AFC, $P < 0.05$.

μg of antigen injected, there is an increase in the total numbers of AFC per spleen, but when the corresponding increase in the numbers of responding ARC is taken into consideration there is not an increase in the number of AFC/ARC in CBA_{T6T6} mouse spleen cells.

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Lower concentrations of SRBC antigens injected into mice have been shown to produce a lower and later peak response of plaque-forming cells than large concentrations of the antigen (17–19). For this reason it was decided that animals receiving 0.5 μg of antigen after irradiation and cell transfer, should be tested on the 9th as well as the 7th day. The results summarized in Table IV indicate that the AFC and ARC response for 0.5 as well as 25 μg POL has reached a plateau by day 7 and are not significantly different from day 9.

ARC Focus and AFC Response Related to Time.—In an effort to determine the optimal timing of the assay and to follow the kinetics of the response of ARC as

TABLE IV

AFC and ARC Responses in CBA_{T6T6} Mouse Spleen Cells Related to Antigen Dose and Day of Assay

Antigen (POL) dose	Day of assay (post antigen)					
	Day 5		Day 7		Day 9	
	AFC	ARC	AFC	ARC	AFC	ARC
μg						
0.5	120		270*		108*	
				0.44		0.66
25	170		400		470	
		1.5		2.5		3‡

* Using the nonparametric rank test, day 7 and 9 are not significantly different.

‡ Only five animals survived to be tested. All other points are the arithmetic mean values of from 10–20 mice.

Each lethally irradiated host received 2×10^6 normal donor spleen cells.

All results expressed as the mean total per spleen.

well as the AFC within them, lethally irradiated CBA_{T6T6} mice which had received 2×10^6 nucleated donors spleen cells, were injected with 25 μg of POL after 24 hr and sacrificed 3–9 days later. The animals were divided into two groups; one was used for the assay of AFC and the other for ARC. Foci were detected first on the 5th postantigen day, and there was a rise in numbers of from 1.1–2.5 foci per spleen by the 7th day (Fig. 8). Most animals did not survive irradiation for more than 9 postantigen days, and of those which survived, about 70% of the spleens exhibited confluence, making the results on that day difficult to interpret.

By days 3 and 4, the numbers of AFC per spleen were well above background and significantly different from the mean number of 170 ± 120 AFC per spleen, detected on the 5th postantigen day. The numbers of AFC per spleen did not increase from day 6 (410 ± 160 AFC/spleen) to day 9, in spite of the fact that

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70% of the spleens became confluent at day 9, suggesting that cell migration or changes in the type of antibody are involved. The data also show that a focus becomes optimally populated with AFC between days 4 and 6, and thereafter there is no statistically significant increase.

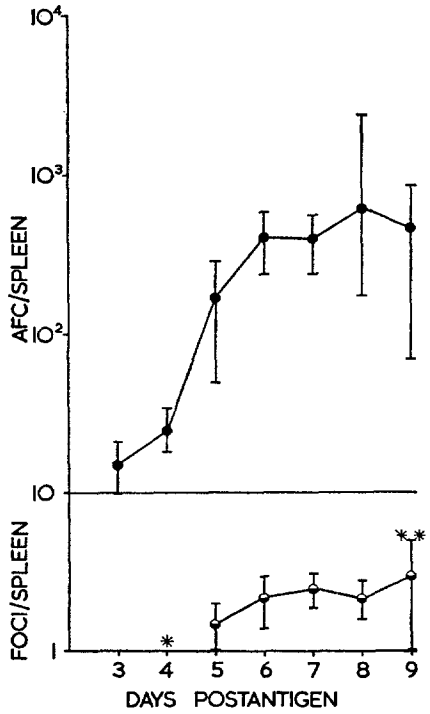

FIG. 8. The ARC focus and AFC response in CBA_{T6T6} mice related to the number of days postantigen that the spleens were assayed. All animals received 800 rads and 2×10^6 spleen cells and 25 μg of polymer antigen 24 hr later.

◒, the arithmetic mean ARC focus response per whole spleen; ●, the arithmetic mean AFC response per whole spleen. The vertical bars indicate 95% confidence limits. The background of 0.05 ± 0.04 foci/spleen has not been substracted from the ARC focus results, but all AFC values are corrected for background. ✳ No foci were detected on day 4. ✳✳, Each point represents the arithmetic mean value for 10–20 animals, except day 9 which, owing to increased mortality and confluence, represents the mean of two animals.

DISCUSSION

The main purpose of this paper is to describe a new technique which permits the enumeration of antigen-reactive cells (ARC) in the spleens of CBA_{T6T6} and $C_{57}BL/Brad$ mice which respond to a well-defined pure protein antigen. The method is based on the belief that when these ARC are injected into a lethally irradiated host, they embed in the spleen in predictable concentrations and respond to an antigenic stimulus by proliferating and differentiating into colo-

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nies of AFC. Such colonies may be detected by virtue of their ability to produce an antibody which immobilizes the indicator bacteria used in the assay.

The sensitivity of the method, as tested by serial dilutions of purified rat IgM and IgG antibody, is equivalent to the antibody-detecting sensitivity of the bacterial immobilization technique (8) and slightly less sensitive than that of the tanned red blood cell technique (14). Since rabbit anti-mouse gamma globulin completely blocks a positive area when directly applied to positive spleen slices, the latter are considered antibody dependent.

To rule out the possibility that serum antibody might be responsible for ARC foci, hyperimmune serum was injected into control animals which had received donor spleen cells shortly after irradiation. The results showed that foci of antibody were present in the spleens of these animals only when the serum antibody titer was 1:640 or greater. Since the mean antibody titer of all test animals was below 1:25 and none reached the critical level of 1:640, the ARC foci detected in the test animals cannot be due to serum antibody. Foci seen in the control animals receiving hyperimmune serum may have been caused by antibody present in splenic blood vessels from which it was able to diffuse into the surrounding agar, or may be due to specific attachment of passive antibody to reticular cells in lymphoid follicles (15, 16).

Using the bacterial immobilization technique in a fluid rather than agar medium permitted the estimation of the numbers of AFC present in an antibody-containing area in the spleen of a test animal. Although the method has not been fully assessed, it has nevertheless shown that such an area contains elevated numbers of AFC when compared with background foci. These experiments have produced what we feel is convincing evidence in support of our original assumption that antibody-dependent foci are associated with colonies of AFC.

One of the most striking aspects about this new method is the extremely low background (0.07 $\pm$ 0.06 foci/spleen) in the spleens of irradiated control animals receiving antigen only. This figure is 50–90% less than the background present in assays using SRBC antigens (1, 3, 4). We have demonstrated that, when using the polymer of *S. adelaide* flagellin antigen, the background is dependent on (*a*) the presence of antigen, (*b*) the amount of irradiation applied to the host, and (*c*) the timing of the antigen injection (24 hr postirradiation being optimal). This latter finding extends observations made by Taliaferro et al. (13) on another antigen system, i.e., before injecting SRBC antigens at least a 24 hr delay after irradiation is necessary to reduce the host response to minimum levels. Thus, background foci present in the polymer system are probably basically different from that of the SRBC antigen, since they are irradiation sensitive and can be elicited only in those irradiated control mice receiving antigen. This fact leads to the conclusion that background foci to POL have no detectable cross-reactivity component as would seem to be the case with the SRBC antigen (17).

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Perhaps the most important single factor supporting the validity of the ARC assay method is the linear correlation between the number of foci and that of donor spleen cells injected into the irradiated host mice. This linear response, which has a slope equal to one and extrapolates through zero, supports the belief that each focus is the result of a single progenitor ARC. The value of 1 ARC focus/1 $\times$ 10^6 injected spleen cells in CBA_{T6T6} mice corresponds with the results produced by Kennedy et al. (1) and Miller and Mitchell (3); on the other hand, our value is twice the number reported by Claman et al. (4) and one-half that described by Playfair et al. (2). The figure of 5% splenic-trapping efficiency of injected cells 24 hr after injection corresponds with previous reports employing the 24 hr delay after cell injection (2) and, using this figure together with the average number of 12 $\times$ 10^7 nucleated cells per spleen, an estimated total of about 2400 ARC responsive to the polymer antigen are present in the spleens of CBA_{T6T6} mice. This is approximately twice the number of cells responding to SRBC as reported by Kennedy et al. (12) and one-half that of Playfair et al. (2). However, if the 5% cell-trapping efficiency figure is applied to the C_{57}BL/Brad mice with the reduced response of 1 ARC focus/5 $\times$ 10^6 nucleated spleen cells, an estimated 500 ARC are present per spleen, indicating a marked difference in the immune potential of the two strains of mice used. If the number of 160 AFC/ARC, as found in the CBA_{T6T6} mouse spleen, is used to calculate the total number of AFC in normal C_{57}BL/Brad mice after priming, it should be about 8 $\times$ 10^4 AFC/spleen, which is close to the peak actually detected by Diener, i.e., a mean of 9.6 $\times$ 10^4 AFC/spleen in normal 6 day primed C_{57}BL/Brad mice (11).

The kinetics of ARC and AFC related to time follows the expected pattern as seen in the SRBC response reported by others (1). However, using POL, there are greater numbers of AFC/spleen in relation to the numbers of donor cells injected, i.e., a mean of 200 AFC/1 $\times$ 10^6 donor cells on the 7th day. Also, this is true for the number of AFC/ARC foci, there being an average of 160 AFC/ARC on the 7th or the peak mean of 280 AFC/ARC on the 8th postantigen day when compared with the SRBC antigen (1, 18). This may be a result of a more vigorous immune response, less migration of AFC from the foci or, what is perhaps more likely, it may be due to the fact that the use of POL allows the detection of IgM and IgG, whereas the hemolysin-dependent assay only detects IgM antibody. The fact that the confluence of ARC foci which occurs on day 9 is not accompanied by an increase in AFC in the spleen suggests a more rapid and/or a more avid antibody production by the cells present in each focus in the later phase of the primary response.

One of the most interesting findings of this work concerns the response of donor spleen cells to varying concentrations of injected POL, which indicates that more ARC are stimulated to respond as the antigen dose is increased from 0.5–5 μg. Concentrations of antigen greater than 5 μg do not stimulate more

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ARC to respond, either because the saturation dose has been reached or because AFC migrate from the spleen once a certain number has been reached. Investigations performed by Sercarz and Byers (19), Wortis et al. (20) and Jerne[4] using the SRBC antigen have shown that the number of plaque-forming cells rises in normal mice when the antigen is increased up to the saturation dose of 4×10^7 SRBC per mouse. If POL is increased from 0.5–10 μg/test mouse as with the SRBC antigen, there is an apparent rise in the number of AFC. However, when the concomitant rise in the number of ARC is considered, the ratio of AFC/ARC actually decreases over this antigen concentration range.

There are two questions which must be considered regarding the interpretation of these data: (*a*) has the peak AFC response to 0.5 μg POL been reached by the 7th postantigen day as it has with 25 μg POL, and (*b*) to what degree does the migration of AFC from the spleen affect the peak numbers of cells? Wortis et al. (20) have shown that the low dose of 4×10^8 SRBC results in a peak response occurring 1 day earlier than the saturation dose of 4×10^7 SRBC. Employing 0.5 and 25 μg concentrations of POL, our data indicate that, by the 7th day, peak numbers of ARC and AFC have been reached and that this response plateaus till at least the 9th day. Opposed to the plateau effect that we have demonstrated, Sercarz and Byers (19), using the SRBC antigen, have reported a fairly rapid drop in the numbers of plaque-forming cells per spleen 2 days after the peak response. Since our assay system is sensitive to both IgM and IgG antibody, the plateau effect may be a result of the presence in ARC foci of cells which produce IgG as well as cells which produce IgM. Consequently, the time at which we enumerate AFC and ARC is not as critical as in the SRBC system, providing it is done within 2 days of the peak response. The migration of cells from the spleen in vivo cannot be controlled or quantitated at present, and therefore is a qualifying factor in our considerations. Nevertheless, the results with POL may be considered as direct evidence that the rise in total numbers of AFC is due to the increased response of ARC. This leads us to the conclusion that ARC respond in an all-or-none fashion to an antigenic stimulus rather than producing progeny which divide more rapidly with increased antigen concentration. If a fall in the number of AFC/ARC does occur, and our data are not enough to show this point clearly, feedback inhibition and cell migration may be the responsible factors.

The main limitation of the assay is the relatively small effective range of cell numbers (1×10^6–4×10^6 spleen cells) which may be used and the fact that such low numbers of cells make it impossible to do parallel studies of serum antibody due to extremely low titers.

The qualities of the polymer antigen have made it possible to use this ARC assay for the investigation of the kinetics of ARC present in lymphoid organs for both the primary and secondary immune responses as well as tolerance.

[4] Jerne, N. K. Personal communication.

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SUMMARY

A new technique for the enumeration of antigen-reactive cells (ARC) responsive to the polymer antigen of *S. adelaide* flagellin (POL) is described for two strains of mice. Foci have been shown to be antibody dependent, may be mimicked by IgM as well as IgG antibodies, and contain specific antibody-forming cells (AFC). The use of POL offers a system unencumbered by relatively high numbers of background foci which, when present, appear to be basically different from those found using the SRBC antigen.

The response of 1 antigen-reactive cell (ARC) focus/1 $\times$ 10^6 CBA_{T6T6} mouse spleen cells is linearly related to the injected number between 1 $\times$ 10^6–3 $\times$ 10^6 donor spleen cells and since 5% of injected cells remain in the spleen, there are an estimated 2400 ARC/spleen.

The number of ARC foci does not increase significantly after the 5th post-antigen day, and by the 8th day the AFC progeny of ARC have reached the maximum mean of 280 AFC/ARC focus. In response to increasing antigen concentrations, an initial rise in the number of AFC as well as ARC is observed, resulting in a relatively constant AFC/ARC ratio. This suggests that the number of ARC stimulated determines the total number of AFC produced under these conditions rather than a variable mitotic rate of the ARC offspring. The main significance of this technique is that it will allow a study of the kinetics of the ARC in the primary and secondary immune response as well as in immunological tolerance.

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