Depressed Cellular Formation of Antibody to *Shigella* Antigens In Vitro by Spleen Cell Cultures from Unresponsive Mice

HERMAN FRIEDMAN

Department of Microbiology, Albert Einstein Medical Center, Philadelphia, Pennsylvania 19122, and Department of Microbiology, Temple University School of Medicine, Philadelphia, Pennsylvania 19141

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Specific antibody plaque-forming cells (PFC) to *Shigella*-soluble antigen did not appear in spleen cell cultures from *Shigella*-tolerant mice, as occurred with similar cultures prepared from normal mice immunized with *Shigella* antigen prior to sacrifice. Cultures from tolerant mice also failed to form serologically detectable amounts of agglutinins in vitro. Exposure of cell cultures from tolerant mice in vitro to additional antigen had little or no effect on appearance of plaque-forming cells to *Shigella*. Spleen cells from normal control mice formed readily detectable levels of antibody, as well as specific antibody plaque-forming cells, after similar stimulation with antigen either in vivo or in vitro. The absence of antibody-forming cells in cultures prepared from spleens of tolerant mice was specific since such cultures, as well as those from normal control mice, formed numerous antibody plaques to unsensitized sheep erythrocytes in vitro after in vivo challenge of the mice with sheep erythrocytes. Tolerance to *Shigella* antigen, as assessed by absence of antibody-forming cells in vitro, persisted for several months. Spleen cell cultures from tolerant mice less than 3 to 4 months of age did not form significant numbers of antibody plaques, even after in vitro exposure to specific antigen. However, spleen cultures prepared from neonatally treated mice, approximately 6 to 8 months old, formed essentially normal numbers of specific PFC in vitro, indicating that the animals had "recovered" from tolerance and that their lymphoid cells were capable of responding to *Shigella* antigen in vitro. Absence of specific PFC in cell cultures from tolerant animals supports the concept that tolerance is due to a central failure of specific immunocompetent cells and not due to an inhibitory effect caused by either "excess" antigen or humoral antibody.

Specific immunologic tolerance or paralysis to a variety of antigens such as serum proteins, simple chemical haptens, foreign erythrocytes, or microbial products is considered to be related to a central failure of antibody formation (13, 20, 22). Results of previous studies in our laboratory indicated that mice injected at birth with a relatively large inoculum of somatic antigens derived from *Shigella paradyseae* become tolerant so that subsequent injection later in life with *Shigella* antigen fails to elicit a typical agglutinin response, as occurs in normal, non-tolerant mice (3, 5, 10). Such unresponsiveness, which is specific and dependent upon dose and route of antigen inoculation at birth, may persist for many months.

Different experimental approaches have been used previously to determine whether specific antibody-forming cells are present in tolerant

animals. Although large numbers of antibody-containing cells could be demonstrated readily in spleens and lymph nodes of control immunized mice by immunofluorescent techniques, only occasional antibody-containing cells were found in similar lymphoid tissue from tolerant mice, either prior to or following challenge immunization with *Shigella* (8). On the other hand, many antibody-forming cells to other antigens, such as *Salmonella*, were detected by immunofluorescent techniques in spleens and lymph nodes of both tolerant and control animals following challenge immunization with the unrelated antigen. However, it is possible that the sensitivity of immunofluorescent techniques may not be sufficient to demonstrate cells which contain very low levels of antibody (8, 16). An indirect antibody plaque technique was utilized previously to detect and localize specific antibody-secreting cells in

lymphoid tissues of *Shigella*-tolerant and normal mice (9). No evidence was obtained that significant numbers of antibody-forming cells to *Shigella* could appear in spleens of tolerant animals after challenge immunization.

In a different approach, lymphoid cells from *Shigella*-tolerant mice have been transferred to normal and X-irradiated recipients, followed by challenge immunization with specific antigen (3, 6). Whereas spleen cells from normal mice transferred specific agglutinin-forming ability, similar cell suspensions from tolerant mice were ineffective. "Adoptive" tolerance was readily demonstrated in X-irradiated recipient mice injected with spleen or lymph node cells from tolerant donors, suggesting that the transferred cell population lacked specific immunocompetent cells (6). In additional studies, maintenance of lymphoid cells from tolerant mice in tissue culture medium under conditions in which cells from normal mice readily formed detectable agglutinins failed to result in significant antibody formation (7). The results of these experiments suggested that the lymphoid cell populations from animals tolerant to *Shigella* antigens could not "escape" from tolerance in vitro.

In these experiments, a similar approach was used, with the addition that the antibody plaque technique was utilized as a sensitive indicator of specific antibody-forming cells in vitro. Spleen cell suspensions from either normal or *Shigella*-tolerant mice were incubated in tissue culture medium in vitro, and the number of specific plaque-forming cells (PFC) to *Shigella* was enumerated at various time intervals thereafter.

METHODS AND MATERIALS

Experimental animals. National Institutes of Health albino A mice were used for these experiments as described previously (8-10). They were obtained from a single colony of mice bred and maintained by a local dealer.

Antigens. *Shigella*-soluble antigen, referred to as SSA, was prepared by trypsin digestion of alcohol-killed suspensions of *S. paradysenteriae* and was used both for induction of tolerance and for in vivo challenge immunization, as described previously (3, 5-10). For in vitro challenge, 1 to 10 μ g of antigen was incubated with spleen cell suspensions from either tolerant or control mice for 2 hr at 37 C, followed by centrifugation and washed twice with cold, sterile medium to remove soluble antigen. In a number of control experiments, both *Shigella*-tolerant and normal adult mice were challenged by intraperitoneal injection of 0.5 ml of a 20% suspension of washed sheep erythrocytes (S-RBC).

Antibody titrations. Serum samples were obtained from control and experimental mice by retro-orbital puncture prior to and at close periodic intervals after challenge injection with antigen. Serial twofold

dilutions were prepared with physiological saline solution, pH 7.2, in microtiter plates in a volume of 0.025 ml, as described previously (7, 9). In addition, supernatant fluids of tissue culture suspensions were also tested for antibody (7). Specific antigen, either alcohol-killed *Shigella*, or washed sheep erythrocytes, was added to each dilution cup, as described in other investigations (7-9). In additional tests, sheep erythrocytes sensitized with SSA were also used (9). In these instances, serological inhibition tests were performed to demonstrate the specificity of the positive hemagglutinin reactions.

In vitro culture technique. Spleens were obtained aseptically from experimental and control mice immediately after sacrifice by cervical dislocation. Cell suspensions were prepared by "teasing" the spleens in sterile medium 199 (Hanks' base, pH 7.2), containing penicillin and streptomycin (100 μ g of each per ml). The cells were washed twice by serial centrifugation in the cold with sterile medium. Viability was estimated by trypan blue-staining technique, and total cell counts were determined with a hemocytometer. The cells were resuspended to 10% by volume in sterile medium. An 0.5-ml of a suspension, with approximately 20×10^6 nucleated cells, was added to 1 or 2 ml of medium containing 20% isologous sterile mouse or fetal calf serum free of serologically detectable *Shigella* agglutinins. Plastic tissue culture tubes, 18 by 150 mm, were used for each culture and were placed in a slowly rotating drum in an incubator at 37 C with an atmosphere of 95% oxygen and 5% CO₂ (7). At least 10 cultures were prepared at a time from a single pool of cells. Prior to incubation and at various time intervals thereafter, 0.1-ml portions were removed from each tube, in duplicate, for determination of the number of antibody PFC. In addition, the medium was usually changed at periodic intervals by gently centrifuging the culture tube and decanting. The supernatant fluids were tested for antibody.

Hemolytic plaque assay. The direct immunoplaque assay for detection of localized zones of hemolysis with uncoated erythrocytes was performed essentially as described previously (4, 9, 12). In brief, 0.1 ml of a suspension of dispersed spleen cells, either obtained directly from an animal or from tissue culture tubes, was mixed with 2.0 ml of warm, melted 0.7% Noble agar (Difco), to which had been added 1.0 ml of diethylaminoethyl-dextran and 0.1 ml of a 10% suspension of sheep erythrocytes. This mixture was carefully layered onto the surface of a previously prepared 100-mm diameter plastic petri plate, containing 15 ml of solidified 1.4% Noble agar in Hanks' solution. Following solidification of the agar (red blood cell-leukocyte mixture), the plates were incubated at 37 C for 1 hr and then treated with 5 to 10 ml of a 1:15 dilution of guinea pig serum as a source of complement. After further incubation for 30 min at 37 C, zones of hemolysis appeared (antibody plaques). The plaques were made more distinct, and the plates were preserved for extended periods of time by staining with a solution of benzidine, hydrogen peroxide, and acetic acid (12). The number of antibody PFC per plate was enumerated, and the number

of PFC per million leukocytes or per culture tube was calculated.

For the indirect or passive antibody plaque technique, sheep erythrocytes treated with *Shigella* antigen were used in place of the untreated red cells, as described previously (9). Washed sheep erythrocytes were incubated for 1 hr at 37 C with 1 to 2 μ g of SSA and then washed several times with saline. The resulting sensitized sheep erythrocytes were used exactly as for the direct plaque procedure. When relatively few plaques were expected with spleen cell suspensions or cultures from tolerant animals, relatively large numbers of cells were used for plating in attempts to obtain counts within the range of 50 to 200 hemolytic zones per plate. In those instances, there were often apparently nonspecific hemolytic areas, probably due to the debris in the suspensions or to aggregated cells. The cell suspensions in these cases were always washed several additional times with saline and filtered through fine gauze. In addition, extra plates were poured in duplicate and not treated with complement, but stained with benzidine. The number of hemolytic zones in these complement-free plates was subtracted from the number of plaques counted in plates treated with guinea pig serum (9). In those experiments in which mice were challenged in vivo with *Shigella* antigen only, or in which tissue cultures were assayed for anti-SSA PFC only, duplicate plates were often tested with unsensitized S-RBC. The number of PFC observed in those plates was subtracted from the number of plaques on plates with sensitized erythrocytes to obtain the calculated number of specific PFC to SSA.

Experimental design. Litters of newborn mice were divided into two groups. One group was either left untreated or injected with saline as a control. The other group was injected with SSA to induce tolerance (10). At various times thereafter, five or more mice from each group were sacrificed, and spleen cell suspensions were prepared. Washed cells were placed in tissue culture medium and incubated for time periods of 5 days or longer, either with or without additional in vitro challenge with SSA (7). Portions of the cell cultures were tested for the presence of antibody-forming cells to *Shigella* by the plaque technique at the various time intervals (9). In addition, both neonatally injected and control mice were challenged with either SSA or sheep erythrocytes at various ages. Several animals from each group were sacrificed 4 or 5 days later, and spleen cell cultures were prepared. The number of antibody-forming cells to either *Shigella* or sheep erythrocytes was determined during a 1-week period in vitro. Also, supernatant fluids of the cell cultures were tested for antibody to these two antigens. Lack of agglutinin formation and absence of antibody-forming cells in the cell cultures from tolerant mice, as compared to agglutinin formation and presence of antibody-forming cells in cultures of normal mouse spleens, produced evidence for continuation of tolerance in vitro.

RESULTS

Antibody-forming cells in spleen cultures from normal and tolerant mice. Normal control mice

and experimental animals injected at birth with SSA were challenged with the same antigen at 3 months of age and sacrificed 4 days later to obtain spleen cell suspensions for culture. Many PFC appeared in cultures from control mouse spleens maintained in vitro for a period of about 5 days (Table 1). In contrast, spleen cell suspensions from tolerant mice formed only occasional plaques. Although the cells were not exposed to antigen in vitro, the number of PFC in control cultures increased during the first few days from an initial level of about 100 PFC per 10^6 leukocytes. Spleen cell suspensions from tolerant mice had about 5 to 10 PFC per 10^6 white cells at the time of sacrifice. There was only a slight increase in the number of PFC during in vitro incubation for several days (Table 1).

The concentration of cells per culture tube seemed to be an important factor for obtaining maximal numbers of PFC in vitro. Although the usual culture contained 20 million cells, other concentrations were also tested (Table 1). Cultures containing about one-fourth as many spleen cells from control mice generally had lower initial PFC counts. There was only a slight to moderate increase in the number of PFC during the next few days in vitro, followed by a rapid decrease on the 4th to 5th day (Table 1). In contrast, when 100 million leukocytes were cultured initially from spleens of normal mice, there were about twice as many PFC as in cultures containing 20 million cells. Also, the number of PFC generally remained higher for a longer time period.

Although a greater concentration of spleen cells from tolerant mice also resulted in slightly more PFC per culture tube, the total number of antibody-forming cells during a 4- to 5-day culture period was still markedly lower than that of control cultures. The maximal number of PFC observed in cultures from tolerant mice was usually less than 15 per 10^6 leukocytes.

Culture supernatant fluids from normal mice challenged with SSA often contained detectable agglutinins to *Shigella* by the 3rd to 6th day of incubation. Titers were generally in the range of 1:16 to 1:32. There were no detectable agglutinins in culture supernatant liquids from tolerant mice at any time.

Viability of nucleated cells, as estimated by dye exclusion tests, remained about 75 to 85% during the first 2 to 3 days in vitro, both for tolerant and control spleen cell suspensions. There was a rapid decline in the total number of cells, as well as the percentage of viable cells, between the 4th to 5th day. Most cultures had fewer than 20% viable cells and less than one-tenth of the original number of leukocytes by the 7th day of culture.

Appearance of PFC in spleen cultures from normal and tolerant mice at various times after in vivo immunization. The time of appearance of antibody plaque formation in spleen cell suspensions from both tolerant and normal mice was studied in detail by the in vitro culture procedures (Table 2). Spleen cell cultures were prepared and the number of PFC was determined at intervals over a period of several days. The maximal number of PFC appeared when the spleen cells were obtained from normal control mice 5 days after antigen challenge. Very few, if any, detectable PFC were observed before the 2nd day. Cultures prepared 8 to 12 days after antigen

challenge had fewer PFC than cultures obtained on the 5th day. Agglutinins were detected in culture supernatant fluids containing cell suspensions prepared from mice 5, 8, or 12 days after immunization.

There were far fewer PFC in cultures of spleen cells from tolerant mice than of control mice regardless of the time of sacrifice (Table 2). The largest number of PFC, usually about 12 to 15 per 10^6 leukocytes, occurred between the 2nd and 3rd day in cultures obtained from tolerant mice 5 days after antigen challenge. The highest serum agglutinin titers, usually several-fold lower than those obtained in normal mice, occurred

TABLE 1. *Number of antibody-forming cells to Shigella in spleen cell cultures from tolerant and normal mice (PFC per 10^6 leukocytes)*

Spleen cell source	No. of cells per culture ($\times 10^7$)	Time in culture					
		1 day	2 days	3 days	4 days	5 days	7 days
<i>Shigella</i> -tolerant ^a	0.81	2.8	8.6	3.4	1.2	2.1	1.0
	2.3	6.5	10.3	8.2	1.3	1.0	1.0
	12.1	15.6	11.2	14.8	10.1	1.0	1.0
Normal immune ^b	0.65	128.6	121.3	113.0	61.2	10.2	2.6
	2.5	364.8	468.8	426.5	316.5	71.2	15.5
	11.2	560.1	682.5	669.2	461.2	114.1	10.2

^a Mice were injected at birth with SSA. They were challenged with SSA at 3 to 4 months of age and sacrificed 4 days later (average serum titer at sacrifice = $<1:10$; average number of PFC/ 10^6 spleen cells at sacrifice = 4.3).

^b Normal 4- to 6-month-old mice injected intraperitoneally with SSA and sacrificed 4 days later (average serum titer at sacrifice = $1:318$; average PFC/ 10^6 spleen cells at sacrifice = 112.8).

TABLE 2. *Effect of time of sacrifice of donor mice on the number of antibody PFC and agglutinin titers in spleen cell cultures from normal and Shigella-tolerant mice injected with SSA at various times before culture*

Spleen cell source	Day after challenge immunization in vivo	Mean peak serum titer	Time in culture ^a				Peak agglutinin titer in vitro
			1 day	2 days	4 days	6 days	
Normal ^b	None	$<1:10$	<1.0	<1.0	<1.0	<1.0	$<1:4$
	2	1:28	43.5	46.5	34.1	13.2	1:8
	5	1:361	96.6	331.2	18.2	18.2	1:165
	8	1:292	43.6	72.9	17.5	10.2	1:209
	12	1:236	12.6	18.5	31.2	3.2	1:112
<i>Shigella</i> -tolerant ^c	None	1:10	1.0	1.3	<1.0	<1.0	$<1:4$
	2	1:11	2.3	1.9	<1.0	<1.0	$<1:4$
	5	1:28	6.1	12.9	5.3	6.1	$<1:4$
	8	1:20	3.6	4.5	4.1	2.5	1:4
	12	1:10	1.5	1.0	1.6	1.2	$<1:4$

^a Cultures initiated with approximately 2×10^7 cells per ml. Number of PFC/ 10^6 leukocytes determined on day indicated.

^b Normal mice 4 to 6 months old injected intraperitoneally with SSA; 3 to 4 mice were sacrificed on day indicated, and spleen cells were pooled.

^c Mice were injected at birth with SSA and challenged at 3 to 4 months of age with SSA; three mice were sacrificed on day indicated, and spleen cells were pooled.

about the 5th to 8th day after antigen challenge. However, there were no detectable agglutinins in culture fluids of tolerant mice, irrespective of the time at which the spleen cells were obtained for culture after antigen challenge.

Effect of *in vitro* challenge with SSA on antibody-forming cells. In the above experiments, there was no *in vitro* challenge stimulation with SSA. For further experiments, cell cultures prepared from 3- to 4-month-old tolerant mice were treated with SSA *in vitro*. There was only a slight increase in the number of PFC in such cultures during a period of 1 to 4 days *in vitro* (Fig. 1). In other experiments, the maximal number of PFC in cultures from tolerant mice treated first *in vivo* and then *in vitro* with SSA was usually less than 20 to 50 PFC per 10^6 leukocytes. In contrast, when cell suspensions were derived from normal mice primed with SSA about 4 weeks prior to sacrifice, there was a vigorous PFC response in tissue cultures to which a small amount of antigen was added on the initial day (Fig. 1). In such experiments with normal mice, there was a background of about 40 to 50 PFC per 10^6 leukocytes at the time of culture. During culture, there was no significant change in the number of PFC unless SSA was added. Exposure

of the cells to SSA for several hours on the first day of culture resulted in approximately a 10-fold increase in the number of PFC during the following 72 to 96 hr (Fig. 1).

Influence of age on continuance of tolerance as determined by *in vitro* culture. Spleen cell suspensions were obtained from either normal or tolerant mice challenged at various ages with SSA. Five or more mice from each age group were bled and sacrificed 5 days after challenge. Spleen cell suspensions were prepared and placed in tissue culture medium. Table 3 indicates the number of PFC per 10^6 leukocytes appearing in the cultures on days 1, 3, and 5. There were few, if any, PFC detected in cultures prepared from tolerant mice challenged with antigen when less than 2 months of age. There were often moderate numbers of specific PFC to *Shigella* in cultures from tolerant mice challenged with antigen at 4 to 5 months of age. Spleen cell cultures prepared from tolerant mice challenged at 6 to 7 months of age had even greater numbers of PFC.

Agglutinin titers of culture supernatant fluids from control mice generally ranged from 1:8 to 1:64. There was usually no agglutinin titer greater than 1:4 or 1:8 in culture supernatant fluids from spleens of tolerant mice between 4 and 6 months of age (Table 3).

Specificity of unresponsiveness *in vitro*. Groups of normal and *Shigella*-tolerant mice were challenged at 3 to 4 months of age with an immunizing inoculum of either SSA or sheep erythrocytes. The animals were sacrificed 4 to 5 days later, and spleen cell suspensions were prepared for culture. SSA was added to one set of tubes. Sheep erythrocytes were added to a second set of tubes, and a third set was treated with both SSA and red blood cells. After 2 hr of incubation, the cell suspensions were washed and resuspended in fresh medium. There were varying numbers of PFC to SSA or to untreated sheep erythrocytes in the various cultures (Table 4). Spleen cell cultures from nontolerant mice challenged *in vivo* with SSA had many PFC to *Shigella*, even without *in vitro* exposure to the antigen. The number of PFC was usually higher when these cultures were treated with SSA. Spleen cells from other control mice immunized with sheep red blood cells alone formed but few PFC to SSA. However, the number of PFC to SSA was often higher in these cultures when treated *in vitro* with SSA than when treated with sheep erythrocytes alone.

There was little or no PFC response to *Shigella* by spleen cell cultures from tolerant mice which were either untreated or injected *in vivo* with

FIG. 1. Antibody PFC to SSA in spleen cell cultures from either normal (○) or *Shigella*-tolerant (●) mice immunized with *Shigella* antigen at 3 months of age. Spleen cell culture prepared several weeks later and treated *in vitro* with 2 μ g of SSA for 2 hr at initiation of *in vitro* incubation. Each point represents average PFC counts of at least five cultures at each time interval tested.

TABLE 3. *Antibody PFC in spleen cell cultures from normal and Shigella-tolerant mice at various ages following challenge immunization in vivo with SSA*

Spleen cell source	Age at challenge immunization with SSA ^a (days)	Antibody response to <i>Shigella</i>				
		Mean peak agglutinin titers in vivo	No. of PFC/10 ⁶ leukocytes in cultures on			Agglutinin titers in vitro (day +5)
			1 day	3 days	5 days	
<i>Shigella</i> -tolerant ^b	14-20	1:11	<1.0	1.8	1.0	<1:4
	25-36	1:28	<1.0	3.3	1.9	<1:4
	48-65	1:19	3.1	7.2	4.5	<1:4
	120-165	1:39	19.3	32.1	30.6	1:4
	195-230	1:183	286.5	331.2	211.3	1:16
	240 or older	1:316	307.6	379.2	206.4	1:48
Normal	14-21	1:193	117.5	185.8	116.8	1:32
	26-33	1:379	279.2	391.1	147.5	1:64
	52-65	1:420	498.1	465.4	310.3	1:48
	85-135	1:408	369.2	423.7	410.8	1:64

^a Five or more mice per group challenged with SSA on day indicated and sacrificed 4 to 5 days later.^b Injected at birth with SSA.

either SSA or S-RBC. Additional exposure of the cultures in vitro to SSA had no effect.

The PFC response to RBC alone was essentially the same for spleen cell cultures from either tolerant or normal mice immunized in vivo with RBC. For example, cultures prepared from *Shigella*-tolerant mice immunized in vivo with red blood cells and treated in vitro with SSA alone had approximately 175 hemolysin-forming cells per million leukocytes, whereas similar cultures from normal mice immunized with RBC had about 150 PFC per 10⁶ leukocytes. When sheep erythrocytes were added in vitro to such cultures, the PFC response to red blood cells increased to about the same level of 450 or 475 PFC per culture. When these cell cultures were treated in vitro with both SSA and RBC, the PFC response to RBC was generally higher than that obtained with RBC stimulation only, suggesting that the *Shigella* antigen exerted an adjuvant effect on cultures from both normal and *Shigella*-tolerant mice.

Cultures prepared from spleens of both tolerant and normal mice challenged in vivo with SSA formed more PFC to RBC following treatment in vitro with SSA. This enhancing activity for SSA on the response to RBC was best observed when both SSA and RBC were added in vitro on the initial day of culture to cell suspensions.

DISCUSSION

The results presented here confirm and extend previous studies, indicating that significant numbers of specific antibody-forming cells to *Shigella* antigen are not present in spleens of *Shigella*-

tolerant animals, as compared to normal controls (9). In addition, previous studies had shown that serologically detectable antibody formation could not be induced in spleen cell cultures from tolerant animals, as occurred with similar cultures from normal animals (13). However, in the previous in vitro experiments, antibody activity to *Shigella* was determined only by serological titrations of culture supernatant fluids or cell extracts.

It seems unlikely that the presence of antigen in cultures prepared from tolerant mouse spleens could interfere with detection of specific antibody-releasing cells, as may occur with free antibody. Since significant numbers of plaques to SSA were not observed in spleen cultures from tolerant mice, it appears that continued unresponsiveness to SSA in vitro is on the cellular level. Such results indicate that tolerance to this antigen is not due to "masking" of immunocompetent cells by "excess" soluble antigen. If such were the case, lymphoid cells from tolerant animals maintained in vitro in an environment essentially free of such soluble antigen, especially after several changes of medium, might recover antibody-forming ability. No "escape" from tolerance was noted in these studies with tissue culture suspensions prepared from spleens of tolerant mice, with or without in vitro stimulation with SSA.

Antibody plaques to SSA-sensitized erythrocytes were detected in large numbers only with lymphoid cell suspensions from immunized control animals. There were only occasional "background" PFC in cultures prepared from normal mice that had not been challenged with SSA in

TABLE 4. *Effect of in vivo and in vitro challenge with SSA or sheep erythrocytes on the number of antibody PFC by spleen cell cultures from normal and Shigella-tolerant mice*

Spleen cell source	In vivo immunizing antigen ^a	In vitro challenge antigen		No. of antibody-forming cells per 10 ⁶ leukocytes specific for ^b	
		SSA	S-RBC	SSA	S-RBC ^c
<i>Shigella</i> -tolerant ^c	SSA	+	—	8.9	14.3
		—	+	4.2	12.3
		+	+	7.5	30.5
	S-RBC	+	—	6.2	173.5
		—	+	2.3	456.1
		+	+	7.2	561.2
Normal	SSA	+	—	429.5	16.2
		—	+	316.5	9.3
		+	+	382.1	36.5
	S-RBC	+	—	14.9	148.5
		—	+	3.2	472.1
		+	+	12.1	549.5

^a Tolerant and normal mice injected at 3 to 5 months of age with either SSA or S-RBC; 4 to 5 mice were sacrificed per group 4 days later, and spleen cells were pooled.

^b The number of PFC per culture determined on the 3rd or 4th day of incubation using both SSA-sensitized RBC and untreated RBC; the numbers of indicated plaques to SSA are corrected for the number of PFC on unsensitized RBC plates.

^c Mice injected at birth.

vivo. Incubation of cell suspensions from normal mice with SSA in vitro failed to elicit a significant PFC response during a culture period of 4 to 5 days. This is in contrast to results reported with other systems, whereby in vitro stimulation of lymphoid cells from normal animals with antigens such as sheep erythrocytes or microbial products, including flagella, apparently induces a rapid immune response (1, 2, 11, 15). However, conditions of culture and antibody assay were probably significantly different between this study and those reported by others.

It seems unlikely that the antibody plaque responses detected in this study in cultures maintained in vitro for varying periods of time could be caused by nonspecific factors unrelated to specific antibody formation. The plaques were complement dependent and could be readily distinguished from the occasional "spurious" plaques often observed in plates containing large numbers of spleen cells. Such artifacts were apparently caused by cellular debris or cell aggregates. Also, appearance of PFC in plates containing spleen cells from control or

tolerant mice would be completely abolished by incorporation of SSA directly into the indicator agar layer. Similar inhibition of passive hemagglutination reactions in vitro has been used extensively as an indicator of the specificity of various serological reactions.

Development of antibody plaques in test plates could also be inhibited by incorporation of undiluted rabbit antimouse γ -globulin into the agar. Similar inhibition of specific plaque formation has been noted by other investigators, with lymphoid cells producing antibody to a variety of antigens (21, 24). Such inhibition has been regarded as evidence of the γ -globulin nature of the lymphoid cell product. Similar anti- γ -globulin serum, in higher dilutions, has been used to facilitate detection of low-efficiency hemolysin-forming cells, presumably due to 7S (IgG) antibody (24). There was little, if any, enhancement of plaque formation with cells from any tolerant mice studied in the *Shigella* system, regardless of antiserum concentration, suggesting that 7S antibody-forming cells to SSA were not present (*unpublished data*).

There were many more "background" plaques to untreated sheep red blood cells in spleen cell cultures from both normal and tolerant mice stimulated in vitro with both S-RBC and *Shigella* antigen than with S-RBC alone. This effect was apparently due to the endotoxic activity of SSA, which also stimulates a moderate increase in "background" PFC to red blood cells in intact animals, either normal or tolerant to SSA. Similar enhancement of the number of background PFC to red blood cells has been noted by other investigators who described "nonspecific" increases in antibody formation due to bacterial lipopolysaccharides (14, 19).

The results presented here seem to be of direct relevance to the question of whether tolerance is related to central failure of the immune mechanism. There have been recent suggestions that some forms of immunological unresponsiveness could be related to a "feedback" inhibition due to specific humoral antibody (17, 18, 23). It has been suggested that a homeostatic control mechanism mediated by antibody suppresses antibody-forming cells directly. Alternatively, serum antibody, even low levels, may "neutralize" antigen and prevent the afferent stimulation of the immune system. Since in this study antibody formation was determined on the cellular level, it seems unlikely that tissue culture suspensions prepared from spleens of unresponsive mice could be suppressed in vitro for any length of time by humoral antibody possibly present in the intact animal. If the observed unresponsiveness was related to low levels of serum antibody,

cell washing prior to culture, as well as medium changes, should have removed the postulated suppressive antibody. The cultures should then have been able to form antibody following in vitro stimulation with additional antigen. However, cultures containing spleen cells from tolerant animals were unable to form specific PFC for periods up to 1 week in vitro, as did cultures from control mice. However, these results do not exclude the possibility that limited antibody being released in vitro might block further responsiveness of the cells upon stimulation with antigen.

The results presented here extend previous observations that there is a marked absence of specific immunocompetent cells in lymphoid tissue of tolerant mice, as demonstrated both by immunofluorescent and cell transfer studies (3-6, 8). However, in those situations the possibility existed that small amounts of antibody could have been released by many antibody-forming cells. The use of the highly sensitive immunoplaque technique, as described here, suggests that it is unlikely that tolerance in this system is related to a quantitative difference of antibody formation by normal numbers of cells. The sizes of antibody plaques were similar, regardless of the source of spleen cell cultures tested. The only discernible difference was in the number of PFC per culture. Thus, results obtained with cell culture procedures make it unlikely that immunological tolerance in this system could be due only to extracellular suppression of potential immunocompetent cells by either circulating antigen or antibody.

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