STUDIES ON THE NONSPECIFIC DEPRESSION OF THE IMMUNE RESPONSE

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The use of radiation, chemicals, and experimental procedures such as thymectomy in studies involving nonspecific depression of the immune response is well known (1, 2). On the other hand, less attention appears to have been given to nonspecific depression of the immune response induced by a great variety of substances of animal, plant, and bacterial origin. These include normal sera (3), plant tissue extracts as described by ourselves and others (4–6), enzymes (7), animal tissue extracts (8, 9), and endotoxins (10).

During the course of our studies (5) on the immunosuppressive properties of the bean extract, phytohemagglutinin (PHA), it was found that some normal sera possessed similar immunosuppressive properties to PHA.

This paper describes the immunosuppressive properties of normal calf and horse sera and particular attention is given to the possible role of macrophages in the kind of immunosuppression described here.

Materials and Methods

Animals.—Animals used in these experiments were C3H and B10.D2 (new) inbred strains of mice.

PHA.—PHA was obtained from Burroughs Wellcome & Co., London, England, as a frozen-dried powder in vials. Each vial contained 50 mg of extract and was reconstituted with 5 ml of distilled water.

Normal Sera.—Sera used in these experiments were calf serum and horse serum; both were obtained from Burroughs Wellcome.

Treatment of Animals with Serum Proteins.—0.4 ml of calf serum was injected intraperitoneally into mice daily from 4 to 7 days prior to immunization. In other experiments, 0.4 ml of calf serum was administered subcutaneously from varying periods up to 21 days prior to immunization. Further experiments entailed intraperitoneal injections of calf serum after immunization. One injection was given 4 hr after immunization, followed by further injections at 24, 48, 72, and 96 hr after immunization.

0.4 ml of horse serum was injected intraperitoneally in mice daily for 7 days prior to immunization.

Immunization.—

Erythrocytes: Mice were inoculated intraperitoneally with 0.3 ml volumes of a 20% suspension of either rabbit or chicken erythrocytes.

Bacterial antigens: Mice were immunized with the following bacterial antigens (Burroughs

Wellcome) via the intraperitoneal route in the volumes listed: (*a*) Agglutinable suspension of *Salmonella london* O. (× 15 concentrated) 0.2 ml diluted 1:2. (*b*) Agglutinable suspension of *Salmonella typhi* H. 0.2 ml undiluted.

Assay of Antisera.—Mice were bled 6 days after immunization. For the assay of bacterial antibodies, doubling dilutions of sera were made up with saline in Medical Research Council Perspex hemagglutinating trays in volumes of 0.1 ml. To each well was added 0.1 ml of undiluted bacterial suspension. The trays were then incubated at 37°C for 1 hr, and the end point of antibody activity was taken as the highest dilution giving macroscopically observable agglutination. In the case of the erythrocyte antibodies, doubling dilutions of sera were made up as above. 0.1 ml of 2% erythrocytes was then added to each well and, finally, 0.1 ml of fresh guinea pig serum was added to provide excess complement. Complement and serum controls were set up to detect possible nonspecific lytic activity of these reagents. The end point of antibody activity was taken as the highest dilution giving at least 50% lysis of the added erythrocytes.

Statistical Evaluation of Results.—The antibody response was expressed as the negative logarithm to the base 2 of the titer. Means, standard deviations, and the significance of differences between control and experimental groups were calculated using the Student's t test (11).

Cellular Changes in the Peritoneal Cavity.—Mice which had received 7 daily intraperitoneal injections of either calf serum or saline were given one intraperitoneal injection of 2 ml of tissue culture medium TC 199 (Glaxo Laboratories, Ltd., Greenford, England). After 10 min, the mice were anesthetized and as much as possible of the TC 199 was recovered from the peritoneal cavity. Total white cell counts expressed as the number of cells per milliliter of peritoneal fluid were obtained using a counting chamber with improved Neubauer ruling, and differential counts were obtained by staining air-dried smears with Jenner-Giemsa stain.

Estimation of the Degree of Phagocytosis of Erythrocytes by Mouse Peritoneal Cells.—In these experiments, calf serum–treated mice and normal mice were given one intraperitoneal injection of 0.3 ml of 20% rabbit erythrocytes. After 1 hr peritoneal cells were obtained as described above by flushing out the peritoneal cavity with 2 ml of TC 199. Total white cell counts and air-dried smears stained with Jenner-Giemsa were prepared as described above. Nonnucleated rabbit erythrocytes were used in order not to confuse total white cell counts which in these experiments were based on counting nuclei. From the stained smears, the percentage of the white cell population engaged in phagocytosis of the injected erythrocytes was estimated. With the value obtained for the total white cell count, the actual number of cells per milliliter of peritoneal cell fluid phagocytozing the injected erythrocytes was determined. For comparison, the degree of phagocytosis of erythrocytes in mice treated with PHA was also determined. Mice were injected daily for 4 days via the intraperitoneal route with 0.4 ml of PHA and treated henceforth in the same way as the mice injected with calf serum.

RESULTS

The Effect of Normal Sera on the Primary Immune Response to Chicken Erythrocytes.—

The effect of administration of normal sera prior to immunization: Seven daily intraperitoneal injections of calf serum (0.4 ml) completely suppressed the formation of detectable hemolytic antibody to chicken erythrocytes when the antigenic inoculum was injected by the same route. In experiments conducted so far, 30 mice injected with chicken erythrocytes without pretreatment with calf serum produced titers of antibody varying from 1:256 to 1:1024, whereas 27 mice similarly immunized but pretreated with calf serum showed no detect-

able antibody. Table I shows that if mice receive four daily intraperitoneal injections of calf serum, the degree of immunosuppression to chicken erythrocytes is not so marked but is still highly significant. The same table indicates

TABLE I

Effect of Normal Sera on the Immune Response to Chicken Erythrocytes in C3H Mice

Treatment of animals	No of animals	The hemolysin response $-\log_2$ titer			SEM	Difference between means of experimental and control groups	Significance of difference of means
		Range	Mean	SD			
		Prior to immunization					
4 daily ip injections calf serum	16	0–7	2.75	2.96	0.740	5.25	$P < 0.001$ ($n = 29$; $t = 6.872$)
4 daily ip injections saline	14	7–9	8.00	0.76	0.202		
7 daily ip injections horse serum	12	0–7	4.67	2.21	0.638	5.04	$P < 0.001$ ($n = 25$; $t = 10.067$)
7 daily ip injections saline	14	9–10	9.71	0.45	0.121		
21 daily sc injections calf serum	15	0–8	4.53	1.09	0.281	4.20	$P < 0.001$ ($n = 29$; $t = 4.448$)
21 daily sc injections saline	15	6–11	8.73	1.48	0.383		
		After immunization					
4 daily ip injections of calf serum commencing 4 hr after immunization	15	9–11	9.93	0.54	0.140	0.19	$P < 0.20$ ($n = 30$; $t = 1.350$)
4 daily ip injections of saline commencing 4 hr after immunization	16	10–11	10.12	0.33	0.083		Not significant

ip, intraperitoneal; sc, subcutaneous.

that seven daily intraperitoneal injections of horse serum also depresses hemolytic antibody formation to chicken erythrocytes but not so markedly as calf serum. If calf serum is administered subcutaneously with the erythrocyte inoculum via the intraperitoneal route, immunosuppression is more difficult to achieve. Seven daily injections had no immunosuppressive action and 10 injections only showed minimal signs of decreased antibody titers. However, when the injections were maintained for 21 days considerable immunosuppres-

sion was evident. (Table I). Similar results were obtained using rabbit instead of chicken erythrocytes.

The effect of administration of normal sera after immunization: Table I shows that treatment of mice with calf serum by a series of intraperitoneal injections commencing 4 hr after immunization, and continuing at 24, 48, 72, and 96 hr after immunization, has no effect on hemolytic antibody formation to chicken erythrocytes.

The effect of normal sera on the primary immune response to bacterial antigens:

TABLE II

Effect of Calf Serum on the Immune Response to Bacterial Antigens in C3H Mice

Antigen used for immunization	Treatment of animals prior to immunization	No. of animals	Agglutination response —$\log_2$ titer			SEM	Difference between means of experimental and control groups	Significance of difference of means
			Range	Mean	SD			
Salmonella london O	7 daily ip injections calf serum	13	5–11	7.77	1.76	0.488	0.38	$P < 0.60$; $n = 25$; $t = 0.582$; Not significant
	7 daily ip injections saline	13	6–10	8.15	1.56	0.433		
Salmonella typhi H	7 daily ip injections calf serum	30	2–8	4.90	1.27	0.233	2.95	$P < 0.001$; $n = 55$; $t = 7.6703$
	7 daily ip injections saline	26	2–10	7.86	1.56	0.306		

ip, intraperitoneal.

TABLE III

*Comparison of the Number of Macrophages and Lymphocytes in the Peritoneal Cavity of Mice Injected With Calf Serum or Saline**

Experimental mice	No. of cells/ml $\times 10^4$	
	Macrophages	Lymphocytes
Saline (1)	12.5	34.0
" (2)	67.2	240.5
" (3)	20.4	91.5
" (4)	45.0	131.5
" (5)	29.5	88.5
Calf serum (1)	214.4	176.4
" (2)	538.2	151.8
" (3)	528.8	264.0
" (4)	165.0	161.7
" (5)	426.5	105.5

* See text for explanation of cell counts.

Table II shows that seven daily intraperitoneal injections of calf serum effectively depresses the formation of agglutinating antibodies to the flagellar antigens of *S. typhi* but not to the somatic antigens of *S. london*. The degree of suppression of the antiflagellar antibodies by calf serum was not so marked as in the case of the hemolytic antibodies.

Effect of normal sera on peritoneal cells: Table III shows that the number of macrophages is considerably increased in mice by injections of calf serum. On the other hand, the number of lymphocytes is not substantially altered by such

TABLE IV

*Phagocytosis of Rabbit Erythrocytes in the Peritoneal Cavity of Normal Mice and Mice Previously Injected With Calf Serum**

Experimental mice	No. of cells/ml $\times 10^4$ containing ingested erythrocytes	
	Less than 5 erythrocytes per cell	5 or more erythrocytes per cell
Saline (1)	9	0
" (2)	4	0
" (3)	3	0
" (4)	8	0
Calf serum (1)	94	6
" (2)	115	13
" (3)	430	92
" (4)	136	28

* See text for explanation of cell counts.

TABLE V

*Phagocytosis of Rabbit Erythrocytes in the Peritoneal Cavity of Normal Mice and Mice Previously Injected With PHA**

Experimental mice	No. of cells/ml $\times 10^4$ containing ingested erythrocytes	
	Less than 5 erythrocytes per cell	5 or more erythrocytes per cell
Saline (1)	30	5
" (2)	84	12
" (3)	24	6
" (4)	8	4
PHA (1)	410	720
" (2)	370	630
" (3)	360	1080
" (4)	540	800

* See text for explanation of cell counts.

treatment. Many of the macrophages in mice treated with calf serum were very large and more vacuolated than macrophages in normal mice.

The effect of calf serum on phagocytosis of heterologous erythrocytes in vivo: Table IV clearly shows that, 1 hr after immunization, the extent of phagocytosis of the injected erythrocytes is considerably increased in the case of mice treated with calf serum compared to normal controls. Many of the phagocytes in calf serum–treated mice ingested large numbers of erythrocytes. Table V shows that similar results were obtained using PHA.

DISCUSSION

These results clearly show that, in the primary immune response, injection of mice with normal calf serum prior to, but not after, immunization considerably suppresses the formation of hemolytic antibodies to chicken erythrocytes. Effective suppression of the immune response to the erythrocytes occurs when both serum and erythrocyte inoculum are injected by the same intraperitoneal route. Administration of erythrocytes by the intraperitoneal route and serum by the subcutaneous route only results in marked immunosuppression if the injections of calf serum are prolonged up to 21 days.

Initially it was thought that the suppression of hemolytic antibody formation might be due to a direct action of serum on erythrocytes. However, incubation of erythrocytes for 2 hr at 37°C with calf serum in no way impaired the ability of the erythrocytes to induce a normal primary immune response in mice. In addition, the demonstration of inhibition of antibody production to some bacterial antigens suggests that, even if calf serum depressed hemolytic antibody formation by a direct action on the erythrocyte inoculum, other properties of calf serum must be of importance in relation to its immunosuppressive activity.

It is also possible to explain these results on the basis of the well-known phenomenon of antigen competition (12). Although the mechanism of this reaction has not been clearly elucidated, it is possible that the injections of calf serum, with concomitant immunization, may have resulted in a paucity of available antibody-producing cells at the time of injection of erythrocytes or bacteria. On the other hand, preliminary experiments have shown that a nonantigenic substance such as starch has similar immunosuppressive properties to calf serum, suggesting that the immunosuppressive properties of calf serum may not necessarily depend on its antigenicity.

The immunosuppressive properties of calf serum are extremely reminiscent of those of PHA described by us elsewhere (5). The similarities between the two immunosuppressants are listed in Table VI. Particularly striking is the fact that both substances suppress antibody formation to erythrocytes and flagellar antigens of *S. typhi* but not at all (or minimally in the case of PHA) to the somatic antigens of *S. london*. These facts suggest that both immunosuppressive agents act by the same mechanism.

The increase in the number of peritoneal macrophages in mice treated with

calf serum or PHA suggests an important role for these cells in this type of immunosuppression. This idea received further support from the demonstration of enhanced phagocytosis by these macrophages. The explanation of the immunosuppressive properties of calf serum and PHA we favor at present suggests that sequestration of the antigen by macrophages in some way prevents the induction of humoral antibody formation. In this context it is interesting to note that high concentrations of newborn calf serum accelerate a formation of hydrolytic enzymes in cultures of mouse peritoneal macrophages (13). In addition, similar enzymic changes have been shown to accompany enhanced phagocytosis in cultures of horse blood monocytes (14). It is possible, therefore, that peritoneal macrophages from mice treated with calf serum or PHA possess

TABLE VI

Comparison of the Properties of PHA and Calf Serum in Mice

Properties	Similarities
Effect on antibody formation to erythrocytes and flagellar antigens of *S. typhi*	Marked suppression
Effect on antibody production to somatic antigins of *S. london*	Minimal or none at all
Effect on macrophage numbers in mouse peritoneal cavity	Marked increase
Effect on phagocytosis of erythrocytes in the mouse peritoneal cavity	Considerably enhanced
Most effective method of obtaining immunosuppression	Injection of both immunosuppressant and antigen by the same intraperitoneal route

increased amounts of enzymes which, by destruction of phagocytosed antigen, prevent antibody formation. Presumably, therefore, the achievement of immunosuppression would depend both on phagocytosis and enzymic destruction of antigen by the peritoneal macrophages. It is suggested that the enzymes of mouse peritoneal macrophages readily destroy the antigenicity of erythrocytes and the flagellar antigens of *S. typhi* but not the somatic antigens of *S. london.* Again, it is of interest to note that peritoneal macrophages have been shown to destroy the antigenicity of erythrocytes (15) but not the antigenicity of the somatic antigens of *Escherichia coli,* a bacterium closely related to *S. london* (16).

SUMMARY

Calf serum has been shown to suppress the primary immune response to erythrocytes and some bacterial antigens in mice if administered prior to and not after immunization. The importance of the route of iniection of antigens and immunosuppressant is indicated. It is suggested that immunosuppression is achieved by phagocytosis and enyzmatic destruction of the injected antigen by peritoneal macrophages. A similar mechanism is probably involved in the type of immunosuppression induced by PHA.

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