The Immunosuppressive Effect of RES "Blockade" with Ethyl Stearate Emulsion on Cellular Formation of Antibody to Sheep Erythrocytes[1] (34488)

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A role for the reticuloendothelial system (RES) in "processing" particulate antigens is regarded by various investigators as a necessary step for the biosynthesis of antibody (1, 2). In this regard, a number of studies during recent years have indicated the importance of unimpaired phagocytosis by RE cells for the production of cellular and humoral antibodies (3–5). Prevention of "normal" uptake of antigen by phagocytic cells apparently results in diminished immunologic responsiveness, as revealed by depressed formation of circulating antibody and specific antibody-forming cells. Most experimental results concerning RES blockade have shown that prior "saturation" of this system by suspensions of iron, carbon, or mineral oil emulsions may interfere with establishment of immunity to a variety of organisms, as well as to sheep erythrocytes (6–10).

Ethyl stearate is one of the ethyl esters of fatty acids which, in emulsion form, markedly affects phagocytic function (10, 11). In the present study, mice were treated with this substance and then challenged with sheep erythrocytes. The ability of the animals to form specific antibody, on the cellular and humoral level, was then determined using the localized hemolytic plaque technique in agar gel.

Materials and Methods. Animals. Male albino NIH mice, 6–8 weeks of age, were used for these studies, essentially as described previously (12). They were housed in groups of five in plastic cages and fed Purina mouse pellets and water *ad libitum*.

Antigen. Freshly washed sheep erythrocytes (RBC), obtained in Alsever's solution, were used (12).

Immunization. Mice were injected intraperitoneally (ip) with 0.5 ml of a standardized RBC suspension, so that each animal received approximately 4×10^8 red cells (12).

Blockading agent. Ethyl stearate [$CH_3(CH_2)_{16}COOC_2H_5$], was obtained from Matheson, Coleman, and Bell. A 50% emulsion of the stearate in physiological saline solution was prepared, using 0.1% Tween 80 as the emulsifying agent. For most experiments, each animal was injected ip with 0.5 ml of the emulsion 1 day prior to immunization. Control animals received 0.5 ml of saline alone, or saline containing 0.1% Tween 80.

Detection of antibody plaque-forming cells. The localized hemolysis in agar-gel technique to enumerate antibody plaque-forming cells (PFC) was used essentially as described previously (12–15). Localized zones of hemolysis which appeared in plates containing cells from immune mice were considered to be due to individual hemolysin-forming cells secreting high-efficiency 19S IgM antibody (15). All plates were stained with benzedine-H_2O_2, and the number of plaques on three or more plates from each cell suspension was determined. The number of PFC's per million leukocytes plated and per whole spleen was calculated (12, 16).

Serology. Mice were bled from the retro-orbital plexus. Individual serum specimens were titrated for anti-sheep hemolysins and

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TABLE I. Effect of Ethyl Stearate Emulsion on Appearance of Hemolysin-Forming Cells in Spleens of Mice Treated 1 Day before Immunization with Sheep Erythrocytes.

Day tested after immunization[a]	Mouse group	No. of mice tested	PFC/10^6 leukocytes	Percent of control	PFC/spleen	Percent of control
1	Control	9	2.9		340	
	Treated	9	1.1	36.8	152	41.3
2	Control	10	6.8		2501	
	Treated	10	2.5	36.3	573	22.9
3	Control	14	52.5		15,903	
	Treated	16	8.6	16.3	2257	14.2
4	Control	14	163.9		58,813	
	Treated	28	76.7	36.3	19,655	33.4
5	Control	13	143.2		55,349	
	Treated	15	122.2	85.3	38,216	69.2
6	Control	9	49.7		18,557	
	Treated	10	33.0	67.0	10,886	58.6
7	Control	5	57.5		21,421	
	Treated	5	42.9	76.2	16,203	75.6

[a] Mice injected ip with 0.5 ml of emulsion 1 day before ip immunization with 0.5 ml 10% sheep RBC's; average PFC response determined on day indicated.

hemagglutinins by standard procedures using microtiter plates and 0.025-ml vol microwire loops (16).

Experimental design. For most experiments mice were treated ip with the emulsion 24 hr prior to immunization with the RBC's. Five or more mice were then sacrificed at daily intervals for the first week after immunization. Control mice treated with saline only were also sacrificed. The number of PFC's in spleens of control and experimental animals was determined. The effect of ethyl stearate on the immune response was evaluated by comparison with the number of PFC's in spleens of untreated controls. In additional experiments, mice were treated daily with the emulsion, with treatment initiated either on the day of immunization, 1 day before, or 1 day after. Other mice were given a single injection of stearate either the same day as immunization or 1 day after. Five or more animals from each group were sacrificed at daily intervals and tested for the number of splenic PFC's.

Results. Control animals had a vigorous cellular immune response to the sheep erythrocytes with a peak on the Day 4 after immunization (Table I). Animals treated with ethyl stearate 1 day prior to RBC injection had, on the average, 60% fewer PFC's (Table I). The percentage of reduction of PFC formation in spleens of stearate-treated animals was even greater during the 3 preceding days, during the exponential rise in the number of antibody-forming cells (Table I). Treated animals tested 1–3 days after immunization had 60–85% fewer PFC's than did the corresponding controls. On Days 5, 6, and 7 after immunization there was only a moderate difference in the number of PFC's per spleen or per million leukocytes between control and stearate-treated animals (Table I).

Animals treated with stearate daily, with treatment beginning 1 day prior to antigenic stimulation, had essentially the same degree of suppression as mice injected only once with the emulsion 1 day before immunization (Table II). There was an average of 55–70% fewer PFC's, either when calculated per spleen or per million leukocytes, on Day 4 after immunization in treated animals, as compared to controls. Mice which received daily injections of emulsion, starting 1 day after immunization, had a moderately enhanced PFC response when tested on Day 4,

TABLE II. Effect of Single or Multiple Injections of Ethyl Stearate, Inoculated before, Simultaneous with, or after Immunization with Sheep RBC's on the Average Number of Splenic PFC's.

Mouse treatment[a]	No. of mice tested	No. of PFC's 4 days after immunization[b] per 10^6 spleen cells	No. of PFC's 4 days after immunization[b] per whole spleen
Stearate treatment			
Daily, starting 1 day before immunization	18	72.1	17,158
Daily, starting 1 day after immunization	12	286.5	82,165
Daily, starting day of immunization	12	131.9	49,361
Single injection day before immunization	14	76.6	19,655
Single injection day after immunization	10	186.2	63,950
Single injection day of immunization	12	148.0	41,360
Controls, no stearate	28	163.9	58,813

[a] Mice injected ip with 0.5 ml of emulsion as indicated relative to day of ip immunization with 0.5 ml 10% sheep RBC's.

[b] Average results of PFC's detected in spleens of indicated number of animals.

or the peak day, after immunization (Table II). There were consistently more PFC's in spleens of these animals at 2 and 3 days, as well as 6 days after immunization. The number of PFC's returned to control values by Day 7. A single injection of ethyl stearate on the day of immunization, or 1 day later had little, if any, effect on the number of antibody-forming cells 4 days after immunization (Table II).

In all experiments mice were bled and their serum specimens tested for anti-sheep cell antibody. There was no significant difference between the titers of control and experimental animals. Mice injected with the red cells, with or without emulsion treatment, generally had a peak hemolysin and hemagglutinin titer 6–10 days after immunization. Titers generally ranged from 1:64 to 1:1024, with an average of 1:662 for the control animals. Treatment of mice with ethyl stearate had no significant effect on the level of hemolysins reached by 5–10 days, or the rate at which the antibody appeared in the serum. The average titer for mice treated with emulsion 1 day before immunization was 1:596, with a range from 1:48 to 1:1024.

Discussion. It is widely recognized that immunosuppression may be achieved by a variety of agents which directly affect antibody-producing cells or their precursors. Such agents include X-irradiation, nucleic acid analogues, and antilymphocytic serum. The results of the present study, as well as earlier studies which utilized carbon as a "blockading" agent (16–18), have indicated that classic RES blockade, induced prior to antigenic stimulation, may suppress the immune response. These results suggest that the common denominator among all RES-blockading agents, as far as the immune system is concerned, may be a possible "competitive inhibition" phenomenon.

Immunosuppression, according to this model, would occur when phagocytic cells, apparently important in the afferent limb of the immune response, become "saturated" with blockading agent and cannot "process" a particulate antigen. However, it should be noted that there have been conflicting reports concerning the effects of RES blockade on immunity. A number of studies have indicated that such substances as carbon, colloidal iron, mineral oil emulsions, or Thorotrast may actually stimulate phagocytosis or immunity (19–21). Other reports, including those from our laboratory, have indicated that similar agents, administered prior to immunization, are effective inhibitors of both cellular and humoral antibody formation (17–19, 22–24). It seems probable that these experimental inconsistencies may be due to

either major or minor differences in such factors as time of administration of blockading agents and/or antigen, dose of antigen and/or blockading material, the type and nature of the antigen, and the prior immunologic status of the individual.

It should also be noted that some reports have indicated that phagocytosis by RE cells may not be necessary for immunity. In particular, Perkin and Makinodan have indicated that "excess" stimulation of peritoneal macrophages, such as those induced by ip injection of oil emulsion, interferes with the immunogenic properties of an antigen (25). It appears either that rapid "digestion" of antigen by stimulated macrophages may result in little or no antibody formation or that phagocytic cells may be capable of degrading macromolecules to nonimmunogenic constituents.

It is also postulated that RES blockading agents may suppress or deplete serum opsonins, which may be important for phagocytosis (26). Thus, if blockading agents suppress the level of the opsonic index, uptake and "processing" of an antigen, such as sheep erythrocytes, may not occur. The nature and function of opsonic antibody in immunity still remains obscure. Inasmuch as the specificity of opsonins is not clear, it is possible that RES blockade by a substance such as ethyl stearate may sufficiently affect the opsonic level to RBC's so that subsequent immunosuppression is only an indirect effect of the emulsion.

In the present study it was found that mice pretreated with an emulsion of ethyl stearate and water had a diminished antibody-plaque response, as compared to nontreated controls, after a single immunizing inoculation of sheep erythrocytes. The maximum depression occurred during the first few days of the immune response. There was an 80–90% or greater reduction in the number of antibody-forming cells in the spleens of emulsion-treated mice tested 3 days after immunization, as compared to controls. The next day, in which the peak response occurred in control mice, there were 65% fewer immunocompetent cells in treated animals. Although the number of antibody-forming cells remained depressed throughout the next few days, as compared to controls, the difference between the two groups became less, on a percentage basis. Also, the peak number of hemolysin-forming cells in treated animals, although much less than in control animals, occurred on Day 5 after immunization. Such results suggest that the ethyl stearate treatment, known to blockade the RE system, probably interfered with the early events of antibody induction and elaboration. The animals appeared to recover competence, with time, and actively formed as much serum antibody as did the controls. The difference between the humoral and cellular results thus probably reflects a significant difference in sensitivity of these two parameters of the immune response, as noted previously with carbon-blockaded animals (16).

Regardless of the actual mechanism whereby RES blockade induces immunosuppression, the results of this study, as well as those previously described concerning the effects of other agents, indicate that substances which alter phagocytosis by RE cells may also markedly affect the immunocompetence of individual cells, either directly or indirectly.

Summary. Mice were injected with an emulsion of ethyl stearate and saline and then challenged with sheep erythrocytes to test their ability to produce specific antibody. Treatment of animals 1 day prior to immunization resulted in a significant decrease in the number of antibody plaque-forming cells to sheep erythrocytes, as compared to control animals. There was approximately a 60–70% decrease in the number of antibody-forming cells on Day 4, or the peak day, after immunization in such treated animals. The immunosuppression was also observable during the first 3 days after immunization, with a maximum depression on Day 3. By Day 5 to 7 after immunization there was less difference between the number of antibody-forming cells in treated and control animals. Multiple daily injections of the emulsion, starting 1 day before immunization, resulted in little additional immunosuppression

over that induced by a single injection. Daily multiple injections starting 1 day after immunization resulted in an enhancement of the antibody-plaque response.

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