ANTIGEN-INDUCED DEPRESSION OF ANTIGEN-REACTIVE CELLS IN IMMUNISED MICE

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THE cellular immune response of mice to sheep red cells (SRC) has been extensively examined by the haemolytic plaque technique of Jerne and Nordin (1963) and Ingraham and Bussard (1964), and by the rosette test of Zaalberg (1964) and Nota *et al.* (1964). In following this response we have found that, under certain conditions, secondary antigenic stimulation consistently causes an initial fall in the number of antigen-reactive cells (ARC). In this paper we report these findings and further observations which suggest that the phenomenon is mediated through some soluble substance resulting from the reaction between antigen and ARC.

MATERIALS AND METHODS

Randomly bred white mice of both sexes were primarily immunised by the intravenous administration of 2×10^8 SRC. The same procedure was employed for secondary immunisation. At intervals thereafter groups of four mice were killed. Their spleens were removed, decapsulated and sieved through a fine stainless-steel gauze into 20-ml volumes of phosphate-buffered saline (PBS) at 4°C. The resulting pooled suspensions were shaken briefly and allowed to stand for 30 min. so that coarse cellular fragments could settle. The suspended cells were transferred to another container, centrifuged for 10 min. at 500*g* and the deposited cells resuspended in 2 ml of PBS. A sample of the concentrated cell suspension was further diluted in 2 per cent. aqueous acetic acid for counting in a haemocytometer. On the basis of this count the suspensions were diluted with PBS to produce suspensions containing 2.5×10^7 spleen cells per ml. These were used for both the Jerne plaque and the rosette technique.

Jerne plaque method. Volumes (5 ml) of 1 per cent. Ionagar in Eison's buffer with 0.02 per cent. DEAE-dextran (Pharmacia) were poured in 9-cm plastic petri dishes to form the base. The same buffered agar, in 3-ml volumes in Universal Containers, was melted and cooled to 46°C in a waterbath; 0.1 ml of neat spleen-cell suspension, or of a 1 in 10 and a 1 in 100 dilution of it when appropriate, was added to each container, followed by 0.1 ml of 10 per cent. washed SRC, mixed quickly and poured into a petri dish as a top layer. The petri dishes were incubated for 1 hr at 37°C, washed for 10 min. with 10 ml of normal saline. For the *direct count*, 5 ml of 1 in 5 fresh guinea-pig serum, previously absorbed with SRC at 4°C, was added and the plates were re-incubated for 1 hr. For *indirect* or *facilitated counts* (Dresser and Wortis, 1965), a 1 in 200 dilution of guinea-pig Fc-specific, anti-mouse gamma globulin was added to the complement. The plaques were counted by naked eye against a good light.

For the *rosette count*, 0.2 ml of 5 per cent. SRC was added to 0.8 ml of spleen-cell suspension, mixed and left at 4°C overnight. The next day the cells were resuspended by gentle agitation and samples diluted with an equal volume of normal saline for counting. Control samples were also incubated for 30 min. with equal volumes of 1 in 10 active and 1 in 10 heat-inactivated guinea-pig serum before counting. All counts were made in a haemocytometer with a green

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filter in the microscope substage. White cells with five or more red cells adherent were counted as rosettes.

RESULTS

Fig. 1 shows the primary response by mouse spleen cells to the inoculation of SRC. Fig. 2 shows the secondary response 52 days after priming with SRC, and fig. 3 the counts obtained after a second challenge. All counts are expressed on a $\log_2$ scale per 10^6 input cells. In each figure, the thick line denotes the direct Jerne count, the thin line the difference between the direct and indirect counts and the broken line the difference between the rosette counts before and after incubation with complement.

Direct Jerne plaques were taken to indicate cells actively secreting lytic antibody of IgM type. In the primary response the direct count rose sharply to a peak on day 4 and fell rapidly to a low level by day 10. In both of the secondarily immunised groups of mice an increase in the direct plaque count occurred. The increase was however lower and of shorter duration than that obtained in the primary response.

The difference between the direct plaque count and the indirect plaque count, as will be discussed later, was considered to reflect the number of cells actively secreting IgG immunoglobulin specific for SRC. For the first 5 days following primary inoculation significant numbers of these cells could not be detected, but thereafter a rise occurred co-incident with the fall in the direct (IgM) count. In the secondarily stimulated groups of mice the rise in the IgG secretory cells occurred earlier, on day 2, and reached much higher levels than in the primary group.

The rosette count was used to detect all ARC, irrespective of the amount or type of antibody they were secreting. These counts invariably exceeded the plaque counts 3- to 4-fold at the peak response and by over 100-fold after an interval of some days following this response. As both groups of primed mice were found to show a fall in the ARC count the day after SRC challenge it was decided to investigate this effect. Challenge with the standard dose of SRC 28 days after priming reduced the rosette and plaque counts the next day by over half. No effect was detected when a tenth (2×10^7 SRC) of the challenge dose was used, or when the standard dose was given only 6 days after priming.

In the next experiment, a group of mice was immunised with two standard doses of SRC given 40 days apart. Four days after the second injection the spleens were removed and suspensions were made to contain 2.5×10^7 cells per ml in Eagle's basal medium without serum; 1-ml volumes of this suspension were dispensed into two tubes; 0.5-ml volumes of 2 per cent. SRC were added to one of these tubes and to a third control tube. Additional Eagle's basal medium was added to each of the three tubes to bring the total volume to 5 ml. The tubes were incubated for 3 days at 37°C and the supernatants obtained by centrifugation. Sixteen mice that 33 days previously had received a standard intravenous injection of SRC were divided into groups of four. The first group acted as control. The mice in the other three groups were each given intraperitoneally 1-ml volumes of one of the three supernatants. All the mice were killed the next day and plaque and rosette counts were made. The

FIG. 1.—Log₂ counts per 10⁶ spleen cells in primarily immunised mice, — direct plaque count, — indirect minus the direct plaque count, ---- complement-sensitive rosette count.

FIG. 2.—Log₂ counts per 10⁶ spleen cells in secondarily immunised mice, — direct plaque count, — indirect minus the direct plaque count, ---- complement-sensitive rosette count.

FIG. 3.—Log₂ counts per 10⁶ spleen cells following a second challenge dose of SRC in mice, — direct plaque count, — indirect minus the direct plaque count, ---- complement-sensitive rosette count.

results in table I show that the supernatant derived from the incubation of ARC with SRC substantially reduced both the plaque and rosette counts.

TABLE I
*The effect of the lymphokine-like substance on the plaque and rosette-forming cells of immunised mice**

Material injected intravenously into mice	Plaques per 10 ⁶ spleen cells		Rosettes per 10 ⁴ spleen cells
	Direct count	Indirect minus direct count	
Nil	12	134	240
Supernatant of Eagle's medium+SRC	20	156	150
Supernatant of Eagle's medium+ARC	12	100	144
Supernatant of ARC+SRC in Eagle's medium	3	44	75

* All the mice were given an intravenous injection of sheep red cells (SRC) 33 days before the experiment.

ARC = Antigen-reactive cells in spleen suspension.

In a further experiment, the same supernatants as in the previous experiments were incubated with an equal volume of immune (to SRC) mouse spleen cells (2.5×10^7 cells per ml) for 3 hr at 37°C. The spleen cell suspension alone,

TABLE II
The effect of the lymphokine-like substance on antigen-reactive cells in vitro measured by the plaque and rosette techniques

Mouse spleen cell suspensions* incubated with equal volumes of	Plaque counts per 10 ⁶ spleen cells		Rosette counts per 10 ⁴ spleen cells
	Direct count	Indirect minus direct count	
Eagle's medium	10	65	103
Supernatant of SRC+Eagle's medium	12	68	97
Supernatant of ARC+Eagle's medium	3	56	72
Supernatant of ARC+SRC	4	27	55

* Obtained from mice that had been given an intravenous injection of sheep red cells 33 days previously.

SRC = Sheep red cells; ARC = antigen-reactive cells in spleen suspension.

with fresh Eagle's basal medium, acted as control. The results of rosette and plaque counts obtained with these suspensions are shown in table II. Again a reduction in the counts was obtained with the ARC plus SRC supernatants.

It seemed unlikely that the substance produced by the action of an antigen on the specific reactive cells would affect only the specific system by which it

was produced. An experiment with two antigens was therefore performed. For this purpose, mice were given 0.2 ml of an equal-volume mixture of 10 per cent. SRC and 10 per cent. chick red cells (CRC). After an interval of 33 days a group of these mice was challenged with SRC, a second group was challenged with CRC and a third group left as control. All the mice were killed the day after challenge. The rosette and Jerne counts of their spleen suspensions in table III show that the ARC count for *both* specificities falls when challenged with *either* of these antigens. In addition, the dependence of the effect on the interaction of the cells with the specific antigen is supported by the observation that challenge of SRC primed mice with CRC did not reduce the ARC count.

TABLE III
The non-specific nature of the lymphokine-like substance

Mice primed with	Mice challenged with	Plaque counts per 10 ⁶ spleen cells		Rosette counts per 10 ⁴ spleen cells with	
		Indirect minus direct counts with			
		SRC	CRC	SRC	CRC
SRC	Nil	195	n.d.	40	n.d.
SRC	CRC	172	n.d.	46	n.d.
SRC+CRC	Nil	46	5	32	152
SRC+CRC	SRC	28	1	18	55
SRC+CRC	CRC	13	2	20	57

SRC = Sheep red cells; CRC = chicken red cells.
n.d. = Not done.

DISCUSSION

The Jerne plaque technique demonstrates the presence of antibody secreting cells. However, not all SRC-specific secretory cells in any suspension will produce a plaque. There are two main reasons for this failure. Firstly, the amount of antibody released from a secretory cell may be insufficient to sensitise enough red cells to enable lysis by complement to produce a visible plaque. Secondly, the type of immunoglobulin secreted from a cell may not fix complement with the efficiency required to lyse red cells. Cells in the second category may be detected by facilitation (Dresser and Wortis, 1965), when anti-mouse immunoglobulin is added to the complement. Plotz, Talal and Asofsky (1968), who used anti-light-chain serum, facilitated lysis by IgM secretory cells and so demonstrated that not all active IgM secretory cells plaqued by the direct method and therefore not all indirect plaques could be attributed to IgG secretion. We used, however, Fc-specific guinea-pig anti-mouse IgG in the indirect technique, so that the difference between this count and the direct count reflected the number of IgG secretory cells. That IgG secretory cells did not form significant numbers of plaques by the direct method was suggested by an observation on spleen cells 8 days after inoculation. These

cells were developed by the direct method. All of the plaques were marked and their size distribution was noted. The plate was reincubated by the indirect method, whereupon additional plaques appeared. These showed a similar size distribution to that of the direct plaques, but there was no increase in the proportion of large plaques in the marked population.

Lymphocytes possessing specific determinants for antigens on SRC bind these to their surface leading to the formation of rosettes. On all occasions the rosette count greatly exceeded the plaque count, ranging from 3- or 4-to-1 at the height of response to 200-to-1 in the resting immune state. Boyden (1964) showed in guinea-pigs that rosettes could also be formed by the interaction of red cells and macrophages which had cytophilic antibody, γ_2 , bound to their surfaces. However, in our experiments, only lymphoid cells were recognised in the centres of stained rosette preparations. Furthermore, treatment of normal mouse spleen cells with immune mouse anti-SRC sera did not sensitise them for rosette formation. In addition, when a mixture of SRC and CRC was incubated with spleen cells derived from mice immunised with a mixture of both types of red cells, the rosettes contained red cells of one type only. On the assumption that sensitised macrophages would have taken up *in vivo* cytophilic antibody of both specificities, some rosettes would have been expected to bear both types of red cells. Thus, in our experiments, the excess of rosettes over plaque-forming cells cannot be due to the mechanism observed by Boyden in guinea-pigs.

Although IgG is inefficient at sensitising red cells for complement lysis (Humphrey and Dourmaskhin, 1965), in the rosette test—unlike the plaque technique—the proximity of the red cells to the ARC and the small number of red cells involved in the reaction may ensure sufficient sensitisation for complement lysis to take place. A rosette that is destroyed following incubation with complement thus indicates an ARC that has secreted antibody (either IgM or IgG) and sensitised the red cells. Since the majority of the rosettes found in our experiments were complement sensitive, the contribution of non-secretory cells to the rosette count must be assumed to be small. The probable explanation of the difference in the counts is that the rosette technique is a more sensitive indication of cells with antibody-secreting function than the plaque technique. Furthermore, modifications of the plaque technique (Cunningham and Szenberg, 1968) are known to increase the sensitivity three-fold.

By means of the techniques described it has been possible, within certain defined conditions, to show that on the day following a second inoculation of antigen into a primed animal there is a decrease in the number of antibody-forming cells. The failure to demonstrate this reduction during the active early phase of the immune response is probably quantitative, since at that time the presence of large numbers of actively proliferating ARC may mask the fall in numbers. The reduction might be due to a temporary switching-off or to death of some of the ARC. The latter is the more likely, for if it were due to a switching-off of antibody synthesis a reduction in the ARC count would be seen in the plaque count alone and not the rosette count.

The *in-vitro* experiments establish that the phenomenon is mediated by a

soluble substance resulting from the interaction of ARC with specific antigen. Published reports indicate that certain factors that inhibit leucocyte migration are cytotoxic and mitogenic and can be liberated by antigen-activated lymphocytes in delayed hypersensitivity (Kolb and Granger, 1968; Thor *et al.*, 1968; Maini *et al.*, 1969). Dumonde *et al.* (1969) have shown the liberation of these factors, "lymphokines", *in vitro*, by lymphocytes from sensitised guinea-pigs. The substance we have demonstrated in mice is probably a "lymphokine" with cytotoxic activity. Such a cytotoxin might be effective not only against target cells, but also against some of the cells that are producing this substance or against other lymphoid cells near the site of its release. Thus the substance, although produced by a specific antigen-lymphocyte reaction, is capable of acting on other cells, as we have demonstrated in mice primed by two antigens. So far we have not attempted to characterise this substance.

The phenomenon could be elicited only by arranging very special conditions. The mice had to be in the "resting" immunological state. A relatively large dose of antigen was required by the intravenous route. Under these circumstances, the antigen would have the best chance of presenting itself quickly to a substantial proportion of ARC. In clinical immunisation procedures the dose and route of administration would make the manifestation of this phenomenon unlikely. In our experiments the effect was transitory, but we believe that the release of this substance might be one of the factors involved both in the induction of high-dose-specific immune tolerance and in the phenomenon of antigenic competition.

SUMMARY

Counts of antigen-reactive cells, obtained by means of the Jerne plaque and rosette techniques, in mice primed with sheep red cells, showed an initial fall after challenge with sheep red cells. To obtain this result, the challenge dose had to be relatively large and to be given at a sufficient interval of time after priming. The reduction in the numbers of antigen-reactive cells is mediated by a soluble substance released in consequence of the interaction of antigen and antigen-reactive cells. The activity of this substance is not restricted to the specific system that led to its formation, since a fall in the count of antigen-reactive cells of a different specificity could be demonstrated in mice primed with two antigens. Though not characterised, the substance responsible for this effect is probably a "lymphokine" possessing cytotoxic activity.

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