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Further Studies of Antigen Stimulation of Deoxyribonucleic Acid Synthesis in Rabbit Spleen Cell Cultures

II. THE EFFECTS OF SPECIFIC ANTIBODY

G. Harris

Department of Experimental Pathology, University of Birmingham

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Summary. Insoluble immune complexes, prepared with heterologous protein antigens and homologous antibodies, affected the incorporation of [^{3}H]thymidine into spleen cell suspension from rabbits immunized to these proteins. Low concentrations of such complexes stimulated thymidine uptake, this being antigen specific, while high concentrations impaired the response to antigen. The inhibitory effect of high concentrations of immune complexes was found to be non-specific as far as the responses to antigens were concerned, but the PHA-responsiveness of rabbit spleen cells was not significantly impaired by the presence of such complexes.

When macrophages from the peritoneal cavities of normal rabbits were incubated with insoluble immune complexes and then washed, such cells were able to stimulate an antigen-specific response by spleen cells from immunized rabbits. It was concluded that, in these experiments, the failure of spleen cells from immunized rabbits to respond to antigen in the presence of immune complexes was the result of a direct inhibition of the mechanism of proliferation by these complexes.

Inhibition of the response to antigen was also produced by the presence of free specific antibodies in the culture medium, even in excess of antigen. It was postulated that, in this situation, antibody reacted with antigen present on the surfaces of cells, particularly macrophages, thus blocking the stimulatory mechanism.

INTRODUCTION

The previous paper (Harris and Cramp, 1968) showed that material sedimented by centrifugation from preparations of heterologous proteins stimulated, *in vitro*, the rate of DNA synthesis in spleen cell suspensions from rabbits immunized to these antigens. The stimulation was antigen-specific and it was found that the deposit from centrifuged protein was more stimulatory than uncentrifuged material or the supernatants obtained after centrifugation. It was concluded that aggregated antigen was present in the sediment obtained by centrifugation and that this would be avidly removed by phagocytosis by macrophages of the reticulo-endothelial system, since it had been shown (Biro and Garcia, 1965) that this occurred with human γ-globulin in intact rabbits, the aggregates being most immunogenic in a primary response to this antigen.

Since the combination of specific antibody with antigen also resulted in removal of circulating antigen at a rapid rate in intact rabbits (Dixon and Talmage, 1951; Weigle, 1958) it was considered of interest to investigate the effects of specific antibody and

immune complexes and compare them with the stimulation of DNA synthesis produced by antigen *in vitro* in spleen cells from immunized rabbits.

MATERIALS AND METHODS

Rabbits

Male and female New Zealand Whites weighing at least 2 kg were purchased from Purdey's Farm, Rochford, Essex.

Antigens

Bovine γ-globulin (Fraction II from plasma) (BGG) was obtained from Armour Pharmaceuticals; human albumin (HSA) and human γ-globulin (Fraction G4) (HGG) were a gift from the Lister Institute of Preventive Medicine.

Immunization

Rabbits were immunized intravenously as described previously (Harris, 1965; Harris and Cramp, 1968) with alum-precipitated antigens.

Phytohaemagglutinin

Bacto-phytohaemagglutinin P (PHA) was obtained from Difco Laboratories Inc., Detroit, and was used at its optimal concentration, for the present system, of 10 μg/culture (Harris and Littleton, 1966).

Isotopically labelled thymidine

[^{3}H]Thymidine at 2–4·0 c/mM, purchased from the Radiochemical Centre, Amersham, was used at a specific activity of 200–300 mc/mM.

Preparation of spleen cell suspensions and macrophages

Spleen cell suspensions and macrophages were prepared from rabbits and cultured by methods already described in detail (Dutton and Eady, 1964; Harris, 1965). For the estimation of the incorporation of [^{3}H]thymidine by cells, 2 μc in 0·2 ml of medium was added during 24–48 hours of culture. The harvested cells were washed twice in ice-cold 5 per cent TCA and then in ice-cold methanol until a colourless precipitate was obtained. Precipitates were dissolved in 0·5 ml hyamine ('hyamine hydroxide', Nuclear Enterprises Ltd, Sighthill, Edinburgh) at 60° for 30 minutes and then added to a standard scintillation fluid for counting in a Packard tricarb, scintillation counter. As in previous publications triplicate cultures were estimated and a variation of more than 15 per cent was not accepted.

Preparation of antigen–antibody complexes

Pooled, heat-inactivated rabbit sera obtained 7–10 days after the termination of a full course of immunization with alum-precipitated antigens were used to prepare specific complexes at equivalence after titration of the antisera (Kabat and Mayer, 1964). The reaction between antigen and antibody was allowed to occur for 1 hour at 37° and then for 48 hours at 4°. The resulting insoluble complexes were then deposited by centrifugation at 1500 rev/min for 45 minutes at 4° and the supernatant tested for the presence of free antibody or antigen which were absent from the preparations used in experiments.

In this way the actual amount of antigen present in the complexes was known and the amount of complex added to the cultures was calculated on the basis of its antigen content. The complexes were washed three times in ice-cold saline immediately before use and experiments were always carried out within 2 days of their preparation, which was always done with preservative-free materials and full sterile precautions. To make soluble complexes a known excess of antigen was added to complexes prepared at equivalence, immediately before addition to cultures.

RESULTS

The effects of specific antibodies to HSA and HGG are shown in Table 1. It can be seen that HSA or HGG in the medium which was supplemented by normal rabbit serum,

TABLE 1

THE EFFECT OF SPECIFIC ANTIBODY

Antigen (μg/culture)	Thymidine incorporation (counts/min/culture)			
	HSA+ NRS	HSA and anti-HSA serum	HGG+ NRS	HGG and anti-HGG serum
0	823	995	823	1126
10	1170	1100	980	995
1000	1493	705	2550	990

Suspensions of spleen cells from a rabbit immunized to HSA and HGG were incubated in medium containing pooled normal rabbit serum (NRS) in the presence or absence of HSA or HGG. Similar cultures were set up using pooled antisera to HSA or HGG instead of normal rabbit serum to supplement the medium, and cells were incubated in the presence or absence of varying concentrations of the specific antigens. The rate of DNA synthesis was measured by the incorporation of [^{3}H]thymidine during 24–48 hours of culture. From calculation of the amounts of antibody present in the cultures, there was excess of antigen at concentrations greater than 100 μg of HSA or HGG/culture.

stimulated the rate of DNA synthesis in spleen cell suspensions from a rabbit immunized to both these antigens and the response was proportional to the concentration of antigen in the cultures. When a fixed amount of specific antibody was present in the medium no stimulation due to antigen was seen at any concentration of HGG, although, perhaps, a slight response to HSA was observed at the lowest concentration of HSA used. Inhibition by specific antibody occurred even in the presence of excess antigen.

From the result of this experiment, it was concluded that specific antibodies inhibit the response to antigens *in vitro*, and further studies were carried out with immune complexes prepared before addition to the cultures, in order to elucidate further the mechanisms of inhibition, with particular regard to whether complex formation played any role.

Fig. 1 shows the effects of three different antigen–antibody complexes used at various concentrations on spleen cells from a rabbit immunized to HSA. Again the relationship between antigen concentration and stimulation of the rate of DNA synthesis was seen. In contrast to the response to HSA it was found that the specific HSA–anti-HSA complexes

were stimulatory at lower concentrations but failed to stimulate at the highest level used. Complexes of HGG with anti-HGG had a similar effect to HSA–anti-HSA complexes. The preparations of HSA and HGG used in these experiments had been found to have antigens in common as shown by double diffusion in agar, therefore spleen cells from rabbits immunized to one preparation of antigen responded, *in vitro*, to the other and this would account for the results shown. This was confirmed by the absence of response to immune complexes with an antigen that did not cross-react with HSA, namely BGG.

The failure of high concentrations of complexes to produce a response to antigen might have been due to a direct effect on the priming of macrophages in the spleen cell suspensions which previous studies (Harris, 1965) had shown to play a role in this *in vitro*

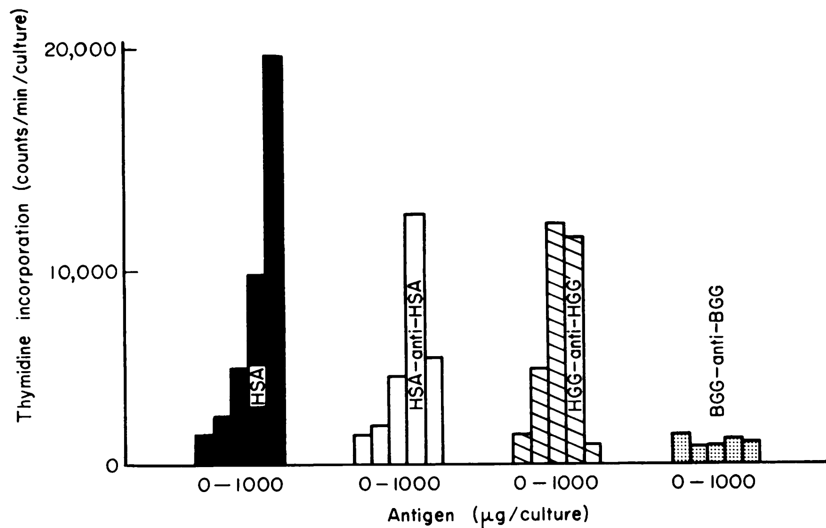

Fig. 1. Spleen cells from a rabbit immunized to HSA were incubated in HSA at 0, 1, 100 and 1000 μg/culture, or together with immune complexes of HSA, HGG and BGG prepared at equivalence and added to cultures at concentrations based on the antigen content of the complexes. The incorporation of [^{3}H]thymidine was measured during 24–48 hours of culture and compared with the incorporation by identical cells incubated in varying concentrations of HSA alone.

response to antigen. The results in Fig. 2 demonstrate that macrophages from the peritoneal exudate of a normal rabbit were primed by exposure to insoluble immune complexes of HSA in a concentration in the medium which failed to stimulate a response when added directly to spleen cells from an immunized rabbit. These primed macrophages stimulated a response in such spleen cells, when cultured together with them, which was as good as the response obtained with antigen added directly to the spleen cells or with similar macrophages primed with antigen alone, at the same concentration as it was present in the immune complexes used for priming.

In the experiment shown in Fig. 3 the response to insoluble complexes prepared at equivalence was compared to responses obtained with soluble complexes prepared in various degrees of antigen excess. No significant loss of stimulatory effect on the rate of DNA synthesis was noted with complexes in antigen excess at the concentration at which insoluble complexes, containing the same amount of antigen, failed to produce a response to antigen. Since the antigen present at each concentration was kept constant for the

different preparations used in this experiment, the ratio of antigen to specific antibody varied and free antigen would be present in the medium with the complexes prepared in high antigen excess, but, presumably, no free antibody would be available.

It was important to ascertain that the effect of high concentrations of insoluble complexes on thymidine incorporation *in vitro* was not inhibitory due to non-specific effects on the spleen cells in culture. Previous studies (Harris and Littleton, 1966) have shown that in spleen cell suspensions from immunized rabbits killed after a boost with antigen 2 days before, cells stimulated to divide by the *in vivo* injection of antigen continued to divide *in vitro* in response to antigen in the medium used to culture the cells. In addition

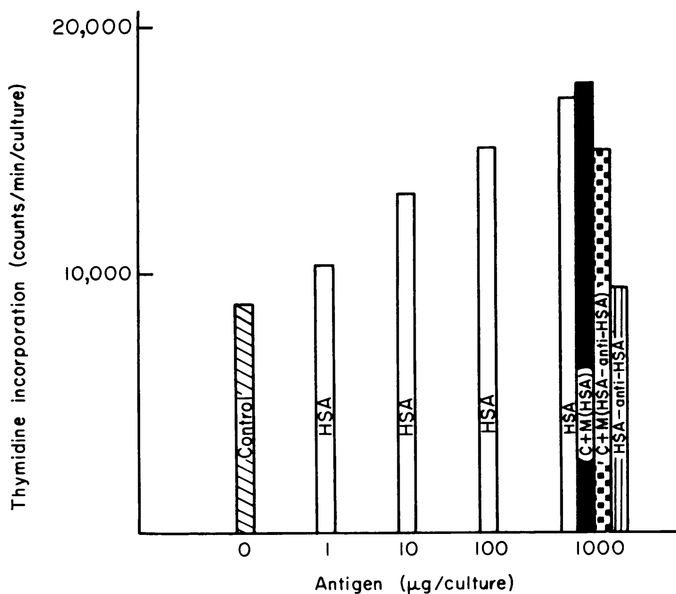

FIG. 2. Triplicate cultures of 5×10^5 peritoneal exudate cells from a normal rabbit were incubated for 12 hours in medium alone, in the presence of HSA at a concentration of 1000 μg/ml [M(HSA)] or with HSA–anti-HSA complexes containing 1000 μg of HSA/ml of medium M(HSA–anti-HSA). The macrophages adhered firmly to the bottoms of the glass culture beakers and were washed three times in pre-warmed medium after overnight incubation. Spleen cell suspensions from a rabbit immunized to HSA were incubated alone (control), in varying concentrations of antigen (HSA), or in the presence of M(HSA) or M(HSA–anti-HSA), and the incorporation of [^{3}H]thymidine was measured during 24–48 hours of culture. The presence of control macrophages had no effect on thymidine incorporation by spleen cells and since these macrophages do not incorporate thymidine (Harris, 1965) these results have been excluded from the figure.

to this population of cells, another population existed which had also been stimulated to divide by *in vivo* immunization but only continued to divide *in vitro* in the presence of PHA. The antigen-responsive cells were found to include those synthesizing antibody while it was concluded that the PHA-responsive cells did not produce antibody under these conditions of culture. The responses of spleen cells from a rabbit which had been immunized to BGG were tested as shown in Fig. 4. The PHA-responsive population of cells was not affected by specific complexes, and while the response to PHA was less in the presence of the non-specific complexes it was still very high as compared to the control incorporation of thymidine. In contrast both specific and non-specific complexes impaired the response to antigen.

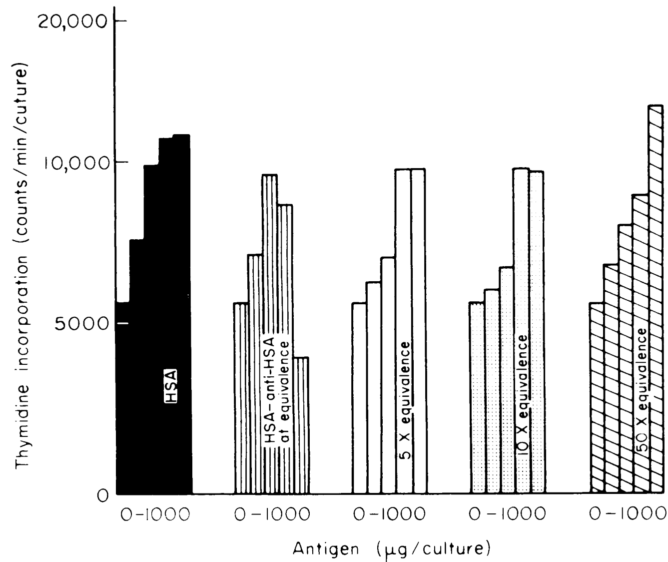

FIG. 3. Spleen cell suspensions from a rabbit immunized to HSA were cultured, in triplicate, in medium alone (control), in the presence of antigen at the same concentrations as in Fig. 1 (HSA), or in specific immune complexes prepared at equivalence (HSA-anti-HSA) or in various degrees of antigen excess. The amount of antigen added to cultures at each concentration was kept constant for the different complexes, therefore the amount of antibody varied inversely with the degree of antigen excess. The cells were incubated in antigen or complexes at different concentrations of antigen and [^{3}H]thymidine incorporation was measured during 24–48 hours of culture.

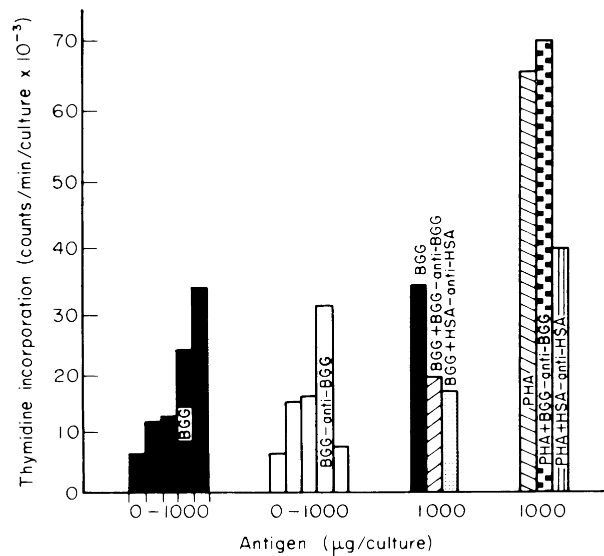

FIG. 4. Triplicate cultures of spleen cell suspensions from a rabbit immunized to BGG were incubated in various concentrations of antigen at the same concentration as in Fig. 1 (BGG) and specific immune complexes containing 1000 μg of antigen/culture (BGG-anti-BGG). Identical spleen cells were also cultured with BGG at 1000 μg/culture or PHA at 10 μg/culture with or without BGG-anti-BGG or HSA-anti-HSA complexes, containing 1000 μg of antigen/culture. The incorporation of [^{3}H]thymidine was measured during 24–48 hours of culture.

DISCUSSION

Previous experiments (Harris, 1965) have demonstrated that specific antibodies can prevent the stimulation of DNA synthesis in spleen cell suspensions from an immunized rabbit by spleen cells from another rabbit which had been incubated in the presence of the antigen and then washed. It was concluded that antibody combined with antigen present on the surfaces of the primed cells and thus prevented stimulation of the cells from the immunized rabbit by this antigen.

In the present experiments, the addition of specific antibody to cultures at the same time as antigen impaired the response of spleen cells from immunized rabbits to the presence of antigen *in vitro*, even when antigen was present in excess. This is in contrast to the failure of complexes prepared in high antigen excess before addition to the cultures, to prevent the response to antigen. Since priming of both spleen cells and macrophages with antigen *in vitro* has been shown to occur very quickly (Dutton and Eady, 1964; Harris, 1965), free antibody could still be available to block stimulation of proliferation in antigen-responsive cells by antigen present on the surface of primed cells. The very slow combination of rabbit antibody, at low concentration, with antigen, taking several days before completion (Kabat and Mayer, 1964) would support this interpretation of inhibition of this *in vitro* response to antigen by specific antibody.

When immune complexes were added to cultures, low concentrations specifically stimulated the rate of DNA synthesis in rabbit spleen cells, while the highest concentration failed to do so. The amount of immune complex formation would appear to be of importance for this effect. When complexes were prepared in antigen excess and added to cultures so that the amount of antigen present was the same at each concentration, but the amount of antibody varied, being least in the region of highest antigen excess (Fig. 4), no loss of stimulatory effect was seen as compared to the results with insoluble complexes which failed to stimulate at high concentration.

In the experiment shown in Fig. 4, complexes prepared in twice antigen excess partially inhibited the optimal response to antigen. The amount of antibody present here was much greater than in the previous experiment and this would result in more immune complex formation.

It has been established (Patterson, Suszko and Pruzansky, 1962) that antigen–antibody complexes prepared near equivalence are taken up *in vitro*, by macrophages in guinea-pig spleen cell suspensions, to a greater degree than complexes prepared in antigen excess, probably because of the larger size of complexes prepared near equivalence. The same holds true for complexes in the circulation of the rabbit (Weigle, 1958). In the present studies complexes prepared at equivalence were able to prime macrophages *in vitro* so that these cells stimulated DNA synthesis in spleen cells from a rabbit immunized to the specific antigen, when both sets of cells were cultured together.

Since no free antibody was present in the medium when insoluble complexes were added to cultures and since macrophages were not apparently affected, the failure of high concentrations of immune complexes to stimulate a specific response to antigen must have been the result of some other effect. The failure of complexes to significantly inhibit the stimulation of DNA synthesis by PHA-responsive cells would rule out the possibility that, in some non-specific way, large amounts of insoluble material interfered with thymidine incorporation by rabbit spleen cells *in vitro*. Further to this, heat-aggregated

proteins have not been found to affect the response of spleen cells to antigens or PHA in culture (personal observations).

If impairment by immune complexes of the response to antigen by rabbit spleen cells *in vitro* were the result of indirect mechanisms, it would be easier to explain the ability of non-specific complexes to reduce the response to specific antigen. Since nothing is known of the way in which contact with antigen results in stimulation of proliferation of responsive cells, any discussion is entirely speculative. One possibility is that immune complexes activated the production of a mitotic inhibitor or chalone which would prevent cell division (Bullough, 1961). The presence of such chemical factors has been demonstrated in such an organ as skin (Bullough, Lawrence, Iverson and Elgjo, 1967). On the other hand, if contact with antigen by specifically responsive cells resulted in activation of a substance (either a product of antigen or something entirely different) responsible for the stimulation of mitotic activity it would be argued that immune complexes might inactivate this stimulatory factor. The failure of complexes to affect the response to PHA would support the idea that PHA acts directly on responsive cells (Ling, 1967 personal communication). From these arguments it would be concluded that the specificity of the impairment of the response to antigen by specific antibodies involves the primary interaction of antigen with antibody either on cell surfaces, blocking the stimulatory effect of antigen on these cells, or in solution, producing immune complexes which themselves interfere with the proliferative response of cells to antigens in a non-specific manner.

The capacity of serum antibody to inhibit specific antibody formation *in vivo* has been demonstrated by many investigations (Smith, 1909; Uhr and Baumann, 1961; Möller and Wigzell, 1965). Most studies in intact animals were concerned with the inhibition of the primary response while the present *in vitro* observations were made on spleen cells from rabbits undergoing a secondary response to antigen. Passively administered antibody to sheep red cells inhibited the primary but not the secondary response to the antigen in rats (Rowley and Fitch, 1964). It has been well established that secondary responses are associated with the production of more antibody-producing cells than primary, therefore it is probable that the secondary response is more difficult to suppress, requiring much more antibody than the primary response to be inhibited in this way.

Current investigation (personal observations) of the *in vitro* response of spleen cells from rabbits immunized to sheep red cells show that only about one cell in every 1000 cells synthesizing DNA in response to antigen is actually producing specific haemolysins. This indicates that only a tiny proportion of the population of cells proliferating *in vitro* in response to antigenic stimulation is actually engaged in specific antibody synthesis. Therefore, although the present studies indicate that the presence of immune complexes *in vitro* impairs the proliferative response to antigen, both specifically and non-specifically, while allowing a good response to PHA to occur, no direct conclusions of the effects on antibody production can be made.

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