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Cyclic AMP and Immune Responses: Changes in the Splenic Level of Cyclic AMP During the Response of Mice to Antigen

[antibody formation/immunoenhancement/immunosuppression/poly(A)·poly(U)]

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ABSTRACT **Intravenous injection of sheep erythrocytes into normal immunologically competent C57Bl/6J mice results in significant and characteristic changes in the splenic level of 3′:5′-cAMP with initiation of the immune response and proliferation of antibody-forming cells. The level increases 2- to 3-fold initially, peaks at 2 min, and returns to base level in an hour. Between 2 and 5 days there is a decrease, followed by a peak when the rate of proliferation of antibody-forming cells is maximal. Changes in splenic level of cAMP are thus transitory and biphasic, and they occur only in response to foreign substances that are immunogenic, such as heterologous erythrocytes, and not to antigenically inert carbon particles. They are also dependent upon the dose of immunogen. Moreover, the double-stranded hybrid of polyadenylate and polyuridylate, which acts synergistically with antigen in stimulating endogenous cAMP, is immunoenhancing if given with sheep erythrocytes when the cAMP level is increasing, and immunosuppressive if given when cAMP is decreasing. These data provide direct evidence for a role of cAMP as a mediator in the activation and proliferation of immunocytes stimulated by antigen. With knowledge of the transitory and biphasic nature of the cAMP response induced by antigen, one can avoid indiscriminate use of drugs that modify the level of endogenous cAMP and instead employ them rationally in controlling the immune response, enhancing or suppressing it as desired.**

With the discovery that cAMP acts as a second "messenger" in regulating hormone-dependent cellular processes, such as differentiation, proliferation, and function (1, 2), attention inevitably turned to the possible role of cAMP in antigen-dependent cellular events. There is now much evidence that cAMP is a protagonist in the activation and functioning of immunocytes responding to antigen (3, 4). The evidence with respect to activation is largely indirect and is based on the effect of stimulators of biosynthesis of endogenous cAMP on the immune response to antigens. One interpretation of this effect is that antigen itself, on reacting with immunocytes, normally stimulates biosynthesis of endogenous cAMP and that stimulators act synergistically with antigen to increase the cellular concentration of cAMP to the level of an activation signal. In this case, one would predict significant changes in the cAMP level of lymphoid organs, such as the spleen, of animals during their response to antigen under physiological conditions. We have been able to verify this prediction by quantitating cAMP in the spleen of mice as they responded to sheep erythrocytes as antigens.

Abbreviations: cAMP, 3′:5′-cyclic adenosine monophosphate; poly(A)·poly(U), poly(adenylic acid)·poly(uridylic acid); PFC, plaque-forming cells; i.v., intravenously; T-cells, thymus-processed cells.

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MATERIALS AND METHODS

Experimental Design. Sheep erythrocytes are injected intravenously (i.v.) into normal immunologically competent mice (C57Bl/6J), and at defined times, ranging from minutes to days, during which the early cellular events in the immune response and the actual proliferation of antibody-producing cells take place, groups of these mice are sacrificed, and their spleens are excised and immediately assayed for cAMP and protein. The splenic level of cAMP is expressed as pmol of cAMP per mg of protein and from these data the profile of its changes can be constructed and related to the immune response.

Preparation of Spleen for Assay. Female C57Bl/6J strain mice (Jackson Labs, Bar Harbor, Me.), 6 to 7 weeks old, were used throughout. At sampling, groups of mice were sacrificed by cervical dislocation, their spleens were excised instantly and immediately homogenized in ice-cold 5% trichloroacetic acid, 8 ml per five spleens. The homogenate was kept in an ice-water bath for 20–30 min, after which a 0.5 ml aliquot was removed for assay of protein and the remainder was centrifuged at 12,000 × *g* for 10 min. One milliliter portions of the supernate were mixed with 100 μl of 1.0 M HCl and trichloroacetic acid was removed by five extractions with 2 ml of water-saturated ether. The aqueous phase was lyophilized and kept for cAMP assay, for which it was dissolved in 300 μl of 50 mM acetate buffer (pH 4.5).

Assay for cAMP. The method of Gilman (5), as modified by Brostrom and Kon (6), was used. The beef binding protein, [^{3}H]cAMP, and protein kinase inhibitor were purchased from Sigma Chemical Co., St. Louis.

Assay for Protein. Protein in the trichloroacetic-acid-precipitated fraction was determined according to Lowry *et al.* (7), with the Folin phenol reagent and bovine serum albumin as standard.

Antibody Response to Sheep Erythrocytes. The Jerne hemolytic plaque assay (8) was used because it is a cellular assay for antibody-producing cells and, therefore, was considered to be best suited to an investigation of the possible relationship between cAMP response and antibody formation at the cellular level.

Poly(A)·Poly(U). This double-stranded hybrid of the homopolymers polyriboadenylate and polyribouridylate is known to be an immunoenhancing agent (9). Purchased from Miles Labs, Elkhart, Ind., it was dissolved in pyrogen-free saline for use.

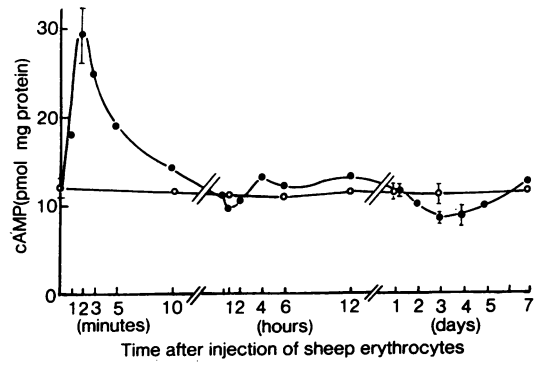

FIG. 1. Changes in level of cAMP in spleens of C57Bl/6J mice during their immune response to sheep erythrocytes. At times indicated, mice given 3 × 10^8 sheep erythrocytes i.v. (●) and controls given physiological saline (○) were sacrificed and their spleens assayed for cAMP and protein. Values are the means of five replicate analyses of pools of five spleens or the mean of analyses of five individual spleens ± SEM.

RESULTS

Changes in spleen cAMP in response to sheep erythrocytes

To determine whether or not the level of cAMP changes in the spleens of mice during immunization with sheep erythrocytes, groups of mice were given 3 × 10^8 sheep erythrocytes i.v. and sacrificed at times ranging from 2 min to 7 days, whereupon their spleens were excised and assayed for cAMP and protein. The results are shown in Fig. 1. Compared to control mice injected with physiological saline, there were significant changes in the splenic level of cAMP occurring at two distinct periods, one within minutes after injection and the second 3–4 days later. The early response resulted in a 2- to 3-fold transient increase in the splenic level of cAMP. The level of cAMP returned to normal by the end of the first hour. In contrast, there was a relatively small decrease in the level of cAMP during the later period, extending over some 3 days.

Temporal relationship between cAMP response and antibody formation

If the cells contributing to the changes in the level of cAMP included immunocytes responding to antigen, one would expect some correlation between the time of cAMP change and specific temporal events in antibody formation, beginning with the activation of antigen-processing and helper T-cells (thymus-processed) and ending with proliferation of activated antibody-producing cells. Each group of animals also included, therefore, mice whose spleens were assayed for antibody-producing plaque-forming cells (PFC). From the results, in Fig. 2, it is apparent that the period during which the cAMP level is decreasing (compare Fig. 1) is coincident with the period of peak proliferation of PFC. The earlier change in splenic level of cAMP seems to be associated with activation of helper T-cells, since Plescia and Hirsch (10) found that functional antigen-carrying T-cells begin to appear in the spleens of mice responding to sheep erythrocytes shortly after the increase in cAMP has occurred. The number of such cells peaked at about 4 hr, while the cAMP increase peaked at about 15 min when sheep erythrocytes were injected intraperitoneally.

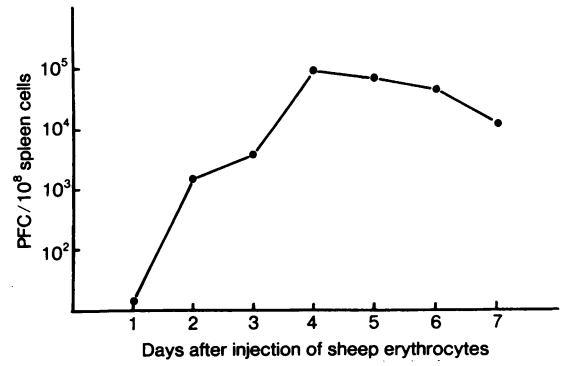

FIG. 2. Kinetics of the immune response of C57Bl/6J mice to 3 × 10^8 sheep erythrocytes, given i.v., in terms of plaque-forming cells (PFC) in the spleen. Groups of four mice were sacrificed at times indicated, their spleens were pooled, and replicate samples were assayed for PFC per 10^8 viable spleen cells.

Characterization of the early cAMP response

Because of the large change in cAMP level observed at 2 min and because this change is very likely due to cells whose type and function in antibody formation are largely unknown, attention was focused on the early cAMP response of mice to sheep erythrocytes.

Dose-Dependence of the Response. Groups of mice were given i.v. injections of 10^6 to 10^9 sheep erythrocytes, and 2 min later they were sacrificed and their spleens were assayed for cAMP and protein. As expected, the magnitude of the change in cAMP was a function of the dose of sheep erythrocytes given (Fig. 3). A plateau was reached at about 10^9 sheep erythrocytes, and a point of inflection in the curve was seen at 3 × 10^8 sheep erythrocytes, which was adopted as the standard dose in subsequent experiments on the interaction of antigen with host cells resulting in elevation of cAMP in the spleen.

Specificity of the Response. As a test of specificity, erythrocytes of syngeneic and allogeneic origin were compared with sheep erythrocytes for ability to stimulate biosynthesis of endogenous cAMP. Control mice were given diluent. At 2 min,

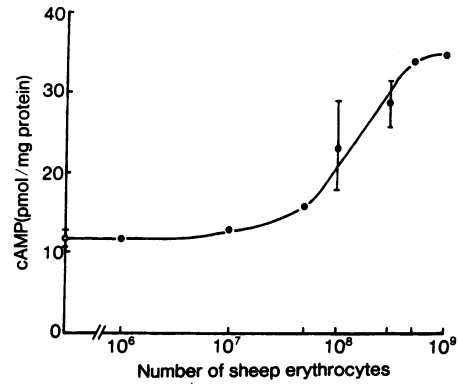

FIG. 3. Change in the level of cAMP in the spleens of C57Bl/6J mice, 2 min after being given sheep erythrocytes i.v., as a function of the number of sheep erythrocytes injected. The values for experimental mice given sheep erythrocytes (●) and control mice given physiological saline (○) are the means of analyses of 10 to 12 individual spleens ±SEM or the mean of analyses of five replicate samples of pools of five spleens.

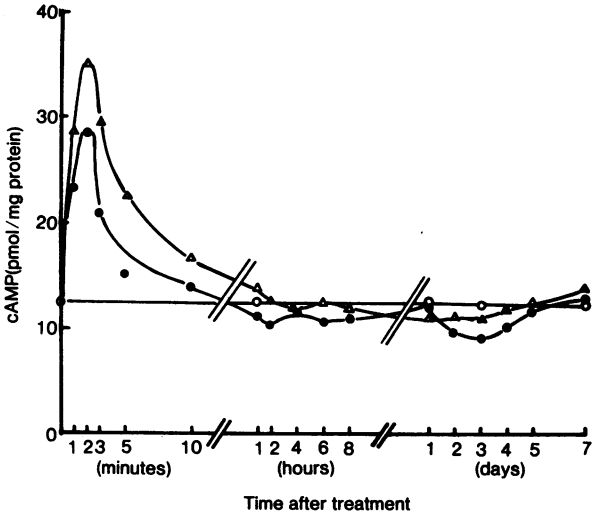

FIG. 4. Effect of poly(A)·poly(U) on the response to cAMP of C57Bl/6J mice. Two groups were given 10^8 sheep erythrocytes i.v., and immediately before this one group received poly(A)·poly-(U) (300 μg i.v.), Δ, and the other diluent, ●; controls received only diluent both times, O. At different times after injection, groups of 5 were sacrified and their spleens were excised and assayed for cAMP and protein. Spleens of mice in a group were pooled and analyzed in quintuplicate; each point is the mean value.

all mice were sacrificed, and their spleens were assayed for cAMP and protein. The results, in Table 1, show that no significant elevation in cAMP was induced by mouse erythrocytes. Apparently, the change in splenic level of cAMP results from the reaction of host cells with foreign substance, the extent of change being a function of the degree of foreignness or antigenicity relative to the host.

Antigenicity as a Factor. The question whether antigenicity might be a critical property and essential for a foreign substance to induce a cAMP response was considered next. Carbon particles, which are not antigenic but are nevertheless cleared rapidly from the circulation by phagocytic cells, were tested for their ability to alter the splenic level of cAMP during active phagocytosis. The data are not given in detail, as no significant change in the splenic level of cAMP was observed.

TABLE 1. *Specificity of the change in the level of cyclic AMP in spleens of C57Bl/6J mice in response to sheep erythrocytes*

Species of erythrocytes (3×10^8, i.v.)	cAMP (pmol/mg of protein) 2 min after injection of erythrocytes
Sheep	26.5
Mouse (C57Bl/6J)	13.4
Mouse (DBA)	13.7
Mouse (Balb/c)	13.2
Saline control	12.1

Groups consisting of five mice were injected with erythrocytes from each species. Two min after injection all animals were sacrificed and their spleens were excised for assay of cAMP. Each value is the mean of 5 aliquots of a pool of the spleens in the group.

Effect of poly(A)·poly(U) on cAMP response and antibody formation

If, as the above data indicate, the changes in endogenous cAMP are due to antigen-reactive cells participating in the immune response to sheep erythrocytes, it follows that agents such as poly(A)·poly(U), which stimulates the antibody response to sheep erythrocytes (9), might affect the cAMP response. This proved to be true; addition of poly(A)·poly(U) to sheep erythrocytes resulted in greater elevation of the cAMP level in the spleen at 2 min (Fig. 4). The poly(A)·poly-(U) seems to act synergistically with sheep erythrocytes in increasing the level of cAMP because poly(A)·poly(U) alone produces no significant change (data not shown).

In view of this finding, and assuming a relationship between the decrease in cAMP and proliferation of antibody-inducing cells starting on day 2, it could be predicted that poly(A)·poly-(U) would be immunosuppressive if given on day 2 relative to the administration of sheep erythrocytes. This was tested by immunization of groups of mice with sheep erythrocytes and giving poly(A)·poly(U), except for the controls, at different times. Fourteen days after antigen was injected, all mice were sacrificed and their spleens were assayed for PFC. The prediction was proved to be correct (Table 2). Poly(A)·poly(U) stimulated maximally the antibody response when given together with antigen, when the cAMP level in response to antigen was increasing, and it suppressed the antibody response when given when the cAMP level was decreasing.

DISCUSSION

The immune response of mice to sheep erythrocytes, under normal physiological conditions, is accompanied by significant changes in the level of cAMP in the spleen. The relevance of the early cAMP response to immunogenicity is based on the finding that only strongly immunogenic foreign substances induced a significant cAMP response. Autologous and homologous erythrocytes, which are not at all or poorly immunogenic, in contrast to erythrocytes of sheep, horse, and chicken, did not stimulate biosynthesis of cAMP (Table 1 and unpublished results). Also, particulate carbon, which is antigenically inert, but sufficiently foreign to be cleared by phagocytosis, did not induce the early cAMP response. Thus, immunogenicity seems to be a critical factor in the early cAMP response of mice to sheep erythrocytes. From the lack of response of cAMP to carbon particles and the absence of

TABLE 2. *Time of administration of poly(A)·poly(U) as a factor in modifying the immune response of C57Bl/6J mice to sheep erythrocytes**

Poly(A)·poly(U) given at time indicated (hr)	PFC/10^8 spleen cells (day 14)
(Control)	2051
0	3440
4	2802
24	1692
48	1500
96	2494

* All mice, including controls, were given 10^8 sheep erythrocytes i.v. at 0 time, and at times indicated groups of five mice were given 300 μg of poly(A)·poly(U) i.v. On day 14 all mice were sacrificed, and their spleens were pooled and assayed in triplicate for plaque-forming cells (PFC).

competition between carbon and sheep erythrocytes, it is also evident that phagocytic cells are not involved in the response to cAMP, a conclusion supported by the recent report that cAMP is not the chemical mediator of metabolic changes accompanying phagocytosis (11).

Evidence of a link between the late change in the splenic level of cAMP and proliferation of antibody-producing cells was first suggested by the coincidence of the time during which both events occurred (Figs. 1 and 2). More definitive evidence is furnished by the finding that poly(A)·poly(U), which acts synergistically with antigen to stimulate synthesis of endogenous cAMP, is both immunosuppressive and immunoenhancing, depending upon the time of its administration relative to antigen (Table 2). It enhances antibody formation at a time when the cAMP level is increasing, and it suppresses when the cAMP level is decreasing. Both activities of poly(A)·poly(U) are maximal when it is injected at the time the change in level of cAMP is just beginning.

Two features with respect to cAMP characterize the response of mice to antigen. First, the change in level of cAMP, both early and late, is transitory, and second, it is biphasic, since the level increases in the early phase and decreases in the later phase. Teh and Paetkau (12), in studying the effect of adding an inhibitor of cAMP-phosphodiesterase at different times during the *in vitro* antibody response of mouse spleen cells to sheep erythrocytes, indirectly showed the same features. The clear implication of these characteristics is that agents that stimulate biosynthesis of cAMP or inhibit cAMP-phosphodiesterase can be both immunoenhancing and immunosuppressive, depending upon the relative timing of their administration, and that such agents should not be used indiscriminately in the control of the immune system. We now can understand why in previous studies the activity of poly(A)·poly(U), in enhancing the immunity of mice to syngeneic tumors, varied with the timing and number of injections of poly(A)·poly(U) given (W. Braun and O. J. Plescia, unpublished results).

Our results now provide direct evidence that antigen itself, without any other external intervention, induces changes in the endogenous level of cAMP which are linked to the activation of the immune system. Moreover, having characterized the transitory and biphasic nature of the cAMP response, we should be better able to exploit drugs that can modify the level of endogenous cAMP, as a means of controlling the immune system.

This research was carried out after the untimely death of Prof. Werner Braun, a long-time colleague and associate of one of us (O.J.P.), but it was his perception of the relationship between immunoenhancing agents, cAMP, and antibody formation that gave direction and impetus to our efforts along a pathway he charted for us as a result of his research, and we are grateful to him for his inspirational guidance.

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