Contract DA-91-591-EUC-[illegible]41

"THE MECHANISM OF ACTION OF ADJUVANTS"

UNIVERSITA' DEGLI STUDI DI MILANO
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MILAN, ITALY
Istituto di Igiene (prof. A. Giovanardi)
and
Clinica del Lavoro (prof. G. Pernis)

F I N A L R E P O R T

March 1st, 1963 - February 29th 1964

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Contract DA-91-591-EUC-2841

Research on: "The mechanism of action of adjuvants"

A B S T R A C T

One year's work on the mechanism of action of adjuvants has been centered on the effects of endotoxins on various aspects of the antibody response. This has been done with experiments on rabbits and experiments on mice.

The experiments on rabbits have shown that endotoxins exert an adjuvant effect on the early stages of the antibody response, inducing an increase mainly of 19S antibodies; when the animals were immunized with conjugated hapten it was noticed that the adjuvant effect of endotoxins resulted in an increase mainly of the antibodies directed against the carrier protein rather than against the hapten itself. A study with histology and immunofluorescence of the spleens of animals 8 days after the immunization with protein antigens and endotoxins showed that the effect of endotoxins was mainly that of increasing the number of large "blast" cells in the periarteriolar sheats and, even more so, in the germinal centers, with considerable decrease of the number of small lymphocytes; most of the new cells appeared to contain 19S gamma globulins.

The adjuvant effect of endotoxins was not significant, instead, in an experiment in which one single dose of endotoxins was followed by a complete course of immunization with protein antigens alone.

In experiments on mice the actual number of cells producing antibodies against sheep red cells (SRC) per mouse spleen, was measured following the agar-plating technique of Jerne and Nordin (1963). The effect of endotoxins, when administered together with the antigen was a considerable increase of the number of antibody-producing cells only in the very early stages of the immune response; later on the animals that had received antigen only "catched up" with those that had also received endotoxins.

In the spleens of untreated mice, in confirmation of earlier work of Jerne and Nordin, a few cells producing anti-SRC antibodies were detected; the number of these increased greatly after administration of endotoxins alone. Because a variety of antigenically different endotoxins were equally effective and because anti-SRC antibodies that appeared in the serum could not be absorbed by high amounts of the endotoxin used, it was concluded that the effect observed with endotoxin alone was not due to antigenic determinants common to endotoxins and SRC, but to a non specific stimulation of cells producing "natural" antibodies.

The histological examination of the spleens of mice revealed an endotoxin effect on lymphoid cells similar to that already observed in rabbits, namely a considerable increase in size of the germinal centers of the follicles with complete or almost complete disappearance of the halo of lymphocytes.

From the work done it is concluded that the adjuvant effect of endotoxins is due to a direct "non specific" effect on the immunologically competent cells, which brings to a large increase in number of immature, 19S producing, "blast" elements; this increase in number of the "blasts" appears due to their enhanced mitotic activity and possibly also to transformation of lymphocytes.

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Fourth Quarterly Technical Status Report

Contract DA-91-591-EUC-2841

Research on:"The mechanism of action of adjuvants"

December 1st, 1963 - February 29th, 1964

In the course of months of December 1963 to February 1964, our research on adjuvants has been centered on further studies on the cellular reactions in the spleens of rabbits immunized with antigens with and without endotoxins. These further studies have been designed to investigate whether an administration of endotoxins with the first shot of antigen, followed by repeated injections of the antigen alone, would still show an effect on the cellular reactions in the spleen and on serum antibodies at the end of the complete course of immunization. A comparable condition, in human beings, might be that of a single shot with associate typhoid and tetanus vaccines followed by administration of tetanus toxoid alone.

The experimental set-up has been the following: adult rabbits have been divided into two groups:

A) Controls: 5 mg BSA (Bovine serum albumin) + 5 mg HGG (human gamma globulin) have been injected intravenously every week during 4 weeks; then after an interval of 2 weeks, the animals received a booster intravenous dose of 2.5 mg BSA + 2.5 mg HGG and have been sacrificed 4 days later. Then the serum antibodies to BSA and HGG have been determined by conditioned hemagglutination and the spleens have been examined by:

   a) Hystology, after staining with methyl green-pyronine

   b) Immunofluorescence, detecting gamma globulins with a fluorescein - conjugated goat anti-rabbit gamma globulin antiserum and detecting anti-BSA and anti-HGG antibodies with the "sandwich" technique of Coons et al. (A.H. Coons, E.H. Leduc and J.M. Connolly - J. exp. Med., 102, 49, 1955). In some experiments anti-BSA and anti-HGG antibodies have been detected in the same sections using on the sandwich, rhodamine-conjugated anti-BSA and fluorescein-conjugated anti-HGG.

B) Experimental animals: these animals have been treated exactly as those of group A, but have received 10 microgm of E.coli endotoxin (Difco) intravenously together with the first injection of the antigens.

I - HISTOLOGICAL OBSERVATIONS (methyl green-pyronine)

In the animals that received antigen alone the more prominent change with respect to untreated rabbits, is a moderate increase of the average size of the lymphoid follicles; outside of the lymphocytic mantle of the follicles a well delimited row of pyroninophilic "blast" cells is observed, the pyronine staining of these elements being definitely more intense than that of those in the germinal centers.

The periarteriolar sheats are thicker than normal and many large pyroninophilic elements are present in the outer layers. In the red pulp more or less mature plasma cells are visible in rows or clusters, here clumps (1-6 μ) of intensively basophilic matter (presumably nuclear residues) are also visible.

In the animals that received endotoxins together with the first shot of antigen the two following aspects are different from the picture described above: 1) the lymphoid follicles are larger, because the germinal centers are larger and show more numeroud cells undergoing mitotic division. 2) the periarteriolar sheats are also thicker, mainly due to a higher number of large pyroninophilic cells in their outer layers. No relevant differences are seen in the number or distribution of the plasma cells in the red pulp.

II - IMMUNOFLUORESCENCE OBSERVATIONS -

The detection, by immunofluorescence, of cells containing anti--BSA or anti-HGG antibodies has shown that in the spleen of the rabbits that had received the course of injection of antigens without endotoxin these cells correspond mainly to the pyroninophilic elements found at the periphery of the lymphoid follicles (outside the lymphocyte wall) and in the outer layers of the periarteriolar sheats.

When both anti-BSA and anti-HGG antibodies have been localised

in the same section (as has been accomplished with the "sandwich" technique and with the use of rhodamine-conjugated anti-BSA and fluorescein-conjugated anti-HGG) it has been possible to show that anti-BSA and anti-HGG antibodies are produced by different cells; these, however, are distributed at random so that at the periphery of each lymphoid follicle or amongst the elements of each periarteriolar sheat both anti-BSA and anti-HGG-containing cells are seen. Spleen sections adjacent to those stained specifically for anti-BSA or anti-HGG were stained with fluorescent anti-rabbit GG antibodies (goat): these were fixed by the plasma cells in the red pulp, by the larger elements outside the follicles and in the periarteriolar sheats and also by the cells in the germinal centers.

In the animals that had received endotoxin together with the first shot of antigens the number and disposition of cells containing anti-HGG or anti-BSA was not, at the end of the complete course of immunization, much different from that of the rabbits that had received antigens only; and in fact the two groups of animals did not show significant differences in serum antibody titers. The staining with fluorescent anti-rabbit GG antiserum showed that the considerable enlarged germinal centers of these animals were not as uniformly positive as those of the ones treated with antigens only, instead there were large areas in which the cells of the centers did not appear to contain gamma globulins.

In conclusion the administration of endotoxin together with the first shot of a course of immunization with protein antigens does not seem to affect appreciably the number of antibody-forming cells (and of the serum antibody titers) found at the end of the immunization course; even at this point, however, some effect of the injection of endotoxin is visible in the spleen as evidenced by the increase in size of the germinal centers; many of these cells, however, did not contain specific antibody or even gamma globulins.

Final technical Report

Contract DA-91-591-EUC-2841

Research on: "The mechanism of action of adjuvants"

March 1st, 1963 - February 29th, 1964

I - ANALYSIS OF THE WORK DONE IN THE PERIOD MARCH 1st, 1963-FEBRUARY 29th, 1964

The work has been centered on the mechanism of the action of one particular type of adjuvant, namely the endotoxins of gram-negative bacteria. These adjuvants were known to differ from other ones in the requirements for a precise timing of administration of adjuvant and antigen (Johnson et al.: J. exp. Med., 103, 225, 1956), in their apparent ability to stimulate the production of antibodies only against protein antigens (see again Johnson et al., 1956), and also in some particular aspects of the histological changes seen in the lymphoid tissues after their administration together with an antigen (Ward et al., J. exp. Med., 109, 463, 1959; Kind and Johnson, J. Immunol., 82, 415, 1959). For these reasons it appeared logical to concentrate on endotoxins our first efforts in the research on the mechanism of action of adjuvants.

Following this purpose we have performed experiments on rabbits and on mice.

1) Experiments in Rabbits. These experiments were aimed to study both the action of adjuvant amounts of endotoxins on the earliest stages of the antibody response and the possible long-term effect of an initial administration of endotoxin followed by a prolonged course of immunization with the antigen alone. The details of the experiments having been given in the quarterly reports, only the main findings and the more important comments will be summarised here.

a) Effect of endotoxin on the early stages (8 days after antigen administration) of the antibody response of rabbits to protein antigens and to

conjugated haptens: the serological studies have shown an increase of the antibodies formed to protein antigens; when the animals were stimulated with conjugated haptens the increased amount of antibody formed under the effect of endotoxins appeared to be primarily directed against the carrier protein rather than against the haptenic determinant. In confirmation of earlier work of Bauer and Stavitsky (Proc. Natl. Acad. Sci., 47, 1667, 1961) an increased production of 19S antibodies appeared to be the main effect of endotoxins in this experiment. This was in accord with the findings of immunofluorescence that showed an increase of cells containing 19S gamma globulins in the periarteriolar sheats and, even more so, in the germinal centers of lymphoid follicles in the spleens of rabbits 8 days after the intravenous administration of antigen and endotoxin. Parallel histological examinations showed an increase of pyroninophilic plasmoblasts and (to a lesser extent) of plasma cells in the periarteriolar sheats and medullary cords, and a dramatic increase in size of the germinal centers of the lymphoid follicles. The latter were increased due to intensive mitotic activity of the "blasts" in the centers, while the halo of lymphocytes was much reduced.

From this experiment it appears that the effect of endotoxins on the earliest stages of antibody formation in rabbits is that of stimulating the proliferation of pyroninophilic "blast" cells; in addition the considerable reduction in number of lymphocytes indicates either a destruction of these elements or (more likely) their transformation into "blasts" (for the possibility of this transformation, see Gowans et al., Nature, 196, 651, 1962). These cytological changes are likely to be the basis of the adjuvant effect of endotoxins, observed soon after their administration with the antigen.

Instead no significant effect of endotoxins on serum antibody titers was found in the second experiment in which:

b) Endotoxins were administered to rabbits with the first dose of antigen followed by a prolonged course of immunization with the antigen alone. In this experiment in fact, the immunofluorescence observations performed

on the spleen at the end of the experiment did not show more antibody-forming cells in the rabbits which had received endotoxin as compared with those that received antigen alone. It is likely that the prolonged stimulation with the antigens induced anyhow a maximal antibody response that would tend to minimize the initial advantage of the animals that had received endotoxin together with the first dose of antigen.

2) Experiments in Mice. In the experiments performed on mice the cellular basis of the adjuvant effect of endotoxins was even better demonstrated by actual counting of the number of antibody-forming cells per mouse spleen, the counting being performed with the agar-plating technique of Jerne and Nordin (Science, 140, 405, 1963).

a) When a good dose of antigen (SRC °) was administered together with endotoxins, it was noticed that the number of antibody-forming cells per mouse spleen was much higher than in the controls (treated with antigen alone) 2 days after immunization; later on the differences became much less evident and at the peak of the response (day 4) the controls had almost "catched up". The mouse/SRC system is obviously very different from the rabbit/BSA (°) or rabbit/HGG (°) system inasmuch as the process of immunization is obviously running at a much faster rate and, in addition, a basal (natural) production of some anti-SRC antibodies was present in our mice; however here again it is apparent that the adjuvant effect of endotoxins exerts itself mainly in the earliest stages of antibody formation.

b) The fact that a small number of cells producing anti-SRC antibodies exists in the normal mouse spleen suggested the opportunity of investigating the effect of endotoxin alone. A considerable increase of cells producing "natural" antibodies were seen in the spleens of mice after treatment with a variety of different endotoxins; this effect was considered to be "non specific", i.e. not due to the presence in the endotoxins

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(°) SRC = sheep red cells - BSA = bovine serum albumin - HGG = human gamma globulins

used of antigenic determinants common with SRC, not only because the same effect was seen with all the different (and antigenically not related) endotoxins tested, but also because the small amounts of anti-SRC antibodies that appeared in the serum of mice after treatment with endotoxins only, could not be absorbed even by high amounts of the endotoxin that had been used to elicit them.

The histological changes seen in the spleens of these mice were similar to those already described in rabbits, and consisted mainly in the almost complete disappearance of lymphocytes at the periphery of lymphoid follicles and (in a somewhat less drastic degree) in the periarteriolar sheats, contrasted by a marked increase of the number of pyroninophilic "blast" cells. The increase of the number of "blasts" was responsible for the considerable enlargement of the germinal centers of the follicles, which, by virtue of the disappearance of the halo of lymphocytes normally present around them, came in direct contact with the red pulp.

It is likely that a lymphocyte-"blast" transformation accounted in part for the observed changes; mitotic division of the "blasts" did, however, certainly contribute greatly to the increase in number of these cells and movement of the lymphocytes to other sites of the body could conceivably also account for the disappearance from the spleen. No histological evidence for destruction of lymphocytes was, instead, seen.

The considerable increase in number of cells producing anti-SRC antibodies in the spleens of mice treated with endotoxins only, deserves a comment in itself. We believe that it might have been due both to multiplication, under the "non specific" effect of endotoxins, of the few cells normally engaged in the production of these antibodies, and also to the transformation into cells producing soluble antibodies (presumably plasmoblasts) of some lymphocytes presensitized towards the production of antibodie of this specificity. This "presensitization" might be seen under different points of view, namely it could conform to some "clonal"differentiation of the immunologically competent cells, or it might reflect previous contact of the lymphocytes with "natural" antigenic determinants not dissimilar from those of the SRC.

If the mice are normally in contact with very small amounts of natural" antigens having some determinants in common with those of SRC, ne might also consider the increase in the number of cells producing anti-RC antibodies after the administration of endotoxin alone as a special ase of the adjuvant activity of endotoxins, and this is the explanation nat we tend to favour.

In conclusion, our work supports the possibility that the adjuvant ction of endotoxins on antibody formation is the consequence of a rapid d presumably direct effect on the immunologically competent cells that sults in an increase in number of young "blasts" that either secrete tibodies themselves (mainly of the 19S type) or will later mature into tibody secreting plasma cells. This effect is due to a stimulation of the totic activity of the "blasts", a fact that is particularly evident at the vel of the germinal centers; in addition to this, the possibility that dotoxins also stimulate the transformation of lymphocytes into "blasts" ems also worth of consideration and further investigation. It is apparent at the adjuvant effect of endotoxins exerts itself in the early stages of e antibody response and that later on, provided that sufficient antigen available or that it is repeatedly administered, the effects of the itial dose of endotoxins become less conspicuous.

- IMPLICATIONS FOR FUTURE WORK

The work so far performed on the adjuvant effect of endotoxins opens teresting possibilities for the investigation of the "non specific" aspects the stimulation of the immunologically competent cells. These "non specific" ects, long neglected in comparison with the specific ones induced by anti-ic determinants, have recently been embodied by Talmage and Pearlman Theoretical Biol., 5, 321, 1963) in an interesting theory of antibody mation whereby the net effect of an antigenic stimulation (tolerance, une paralysis or antibody formation) depend from the balance between the cific and the non specific stimulation of the lymphoid cells.

In future work the cellular reactions to the administration of other types of adjuvants (such as Freund's or wax D) with or without antigens, should be confronted with those already established for endotoxins to investigate whether basic similarities or differences exist between the mode of action of these different cathegories of adjuvants. The complete body of knowledge should eventually lead to a classification of adjuvants on the basis of their mode of action, a prerequisite to an efficient utilization of adjuvants in the immunization of humans.

Prof. Augusto Giovanardi

Prof. Benvenuto Pernis

Augusto Giovanardi

Benvenuto Pernis

Note: The manuscripts and/or reprints of the papers concerning the above mentioned research work will be sent as soon as available.

Contract DA-91-591-EUC-2841

A N N E X

1) Summary of personnel utilized during the reporting period:

in addition to the principal investigators (prof. B. Pernis and Prof. A. Giovanardi) the following academical personnel (scientific staff) has been utilized:

1) G. Chiappino, M.D.
2) E. Grosso, M.D.
3) E. Magliano, M.D.
4) G. Gallingani, M.D. and
5) M. Luisa Profeta, M.D.

The technical personnel (technical staff) had included:

1) Miss A. Grippa (full time)
2) Miss L. Forni (part time)
3) Miss L. Ciffo (part time)
4) Mr. I. Gonzaga (part time)
5) Mr. G. Bertani - photographer - (occasional aid)

2) No. of manhours expended by the scientific staff (not including the principal investigators) - approx. 3,500

No. of manhours expended by the technical staff - approx. 3,600

Expenses for consumable supplies = $ 1,550

Property acquired: one fluorescent lamp for Leitz fluorescence microscope (exchanged with previous old lamp).

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