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DEPARTMENT OF THE ARMY
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#205

Anthrax: Biological and Immunological Foundations of Diagnostic and Prophylaxis. 1. Production and Experimental Investigation of Anthrax Allergen - by E. N. Schljachow

(From the Moldan Institute for Epidemiology, Microbiology and Hygiene, Kischinew. Journal of Hygiene, Epidemiology, Microbiology and Immunology, 2, 266-273 (1958) (Czech.)

As is well known, medical science has only limited possibilities at its disposal with which to determine the status of the infection and immunity by means of laboratory methods in connection with living organisms infected with anthrax.

Upon contact of the anthrax pathogen with man or animal, no antibodies develop in their organism. The usual serologic reactions (agglutination, flocculation, complement fixation reaction, etc.) reveal neither post infections nor post vaccinal immunity against anthrax. The only serologic reaction - thermoprecipitation after Accoli - can be conducted only with lifeless material and therefore cannot be used for the diagnosis of the living organism.

Bacterioscopy and bacteriological cultures of the pathogen of cutaneous anthrax (pustula maligna) are unsuccessful without subsequent biological examinations: it must be considered moreover that the number of pathogens in the carbuncle is small (Gamaleja 1940) and that these die off rapidly (Stanischewskaja 1938) or change into atypical forms (Birger 1949). At the beginning of the internal forms of the disease, bacteriological examinations may be conducted only in the septic phase, which usually has an antemortal character already.

The lack of laboratory tests for the determination of immunity against anthrax becomes apparent in attempts to evaluate the efficacy of animal and especially human vaccination. In such a case some test methods either cannot be used at all (e.g. control inoculations) or they are unexact (epidemiological evaluation). For the time being, the immunization of humans with weakened STI vaccine has been conducted in the USSR for many years and on a sufficiently large scale.

The publications mentioned above bear witness of the actuality involved in the search for new, successful methods making a determination possible of the status of infection and the immunity against anthrax by way of the living organisms of man and animals susceptible to this disease.

Certain prospects for the discovery of an immunobiological test for the determination of the degree of specific reactivity in animals and man to anthrax were shown in the utilization of the principle of animal immunization with sterile, edematous fluid developing in anthrax diseases (suggested by Bail in 1904).

In 1927, Houschka, during immunization of horses against anthrax with simultaneous application of filtered edematous fluid from anthrax-infected guinea pigs or rabbits, as well as anthrax bacilli, noted that repeated application of

edematous fluid causes extensive swelling at the point of inoculation of an animal already immune. Houschka ascribed this phenomenon to a special substance in the edematous fluid -- "plasmoreagin".

The possibility suggested itself that the edematous fluid may produce, aside from the protective substance, a substance which evokes an allergy in the immune organism.

For a long time no practical use was found for this observation. Almost twenty years later Balteanu, Toma and Garaguli used Houschka's experiences in 1943 for the production of a preparation for the determination of the degree of infection and immunity against anthrax in animals and man, and this by introducing the allergic skin test. Balteanu and his co-workers used virulent anthrax bacilli in the production of this preparation, which unfavorably influenced the specificity of the allergen and its biological effect on the tested organisms (a large number of parallel reactions and significant occurrence of tissual disintegration).

Our report contains data on a new method for the production of preparations for the determination of the specific status of the infection and immunity of the organism susceptible to anthrax by the introduction of the allergic skin test. At the same time, we list the results of experimental research with utilization of the obtained allergen.

Material and Methods

Bail assumed that the immunizing effect of the edematous fluid was due to the presence of "aggressins" contained therein. Then Sobernheim, in consonance with the assertions of Gruber and Futaki (1907) and Preiszs (1907, 1909), showed the formation of aggressins as being dependent on the capsular substance of the virulent anthrax bacillus. However, later papers by N. Stamatin and L. Stamatin (1936a, 1936b), Sterne (1937) and Ginsburg (1946) demonstrated that a reliable vaccinal immunity also develops in animals and man when avirulent, noncapsular, but edematizing variacions of the anthrax bacillus are used.

Cromartie, Watson, Bloom and Heckly (1947) found not only the immunizing factor in the edematous fluid, but also the inflammatory element connected with the capsular substance which differs from the first in a number of properties. The inflammatory factor contained in the capsule did not confer immunity.

On the other hand, Smith and Keppie (1954) report the discovery of a special toxin, called the "lethal factor", in the plasma of guinea pigs killed by anthrax, following infection with virulent bacilli. The application of this toxin on the organism of healthy guinea pigs caused tissual disintegration and the animal's death. The lethal factor was neutralized against anthrax with peritoneal and thoracal pus (exudate) as well as with hyperimmune serum.

It is obvious that the edematous fluid, obtained with the aid of virulent anthrax rods, is not free of plasma, and, therefore - in agreement with the data Smith and Keppie - contains a certain quantity of the lethal factor, which

stroys the tissues. With due consideration of the previously mentioned investigations, we endeavored, in contrast to Balteanu et al., to obtain an edematous fluid from guinea pigs and rabbits, which is free of all inflammatory substances, originating in the capsules of the virulent microbe (cf. Cromartie, et al.) and also free of toxic lethal factors (cf. Smith, et al.), containing, however, the allergic element. For this purpose we used non-capsular strains of anthrax or those having capsules, but with significant attenuation of their virulence.

Procurement of the Edematous Fluid

a. Animals: Hybrid white mice weighing 20-22 g., guinea pigs 400-500 g., rabbits 1800-2200 g.

b. Edematizing anthrax strains:

(1) Vaccinal strain STI-1 for animals (non-capsular).

(2) Zenkowsky's second vaccine (bacilli with attenuated virulence, with capsule). Both preparations came from indigenous biological productions and served practical purposes.

c. Animal infection for the production of edematous fluid: the following were instilled in guinea pigs:

(1) Vaccine STI-1 subcutaneously in a dose of 0.1 ccm per 100 g. of the animal's weight. This dose caused swellings in the majority of the animals and death of about 30 percent of the guinea pigs at the highest stage of the swelling on the 3rd to 6th day.

(2) Zenkowsky's second vaccine was administered in the same manner and quantity and caused a general formation of swellings and the death of the animals in all cases on the 2nd or 3rd day. Rabbits were infected subcutaneously with Zenkowsky's vaccine in a dose of 0.1 ccm per 100 g. body weight. In all cases swellings and in 80 percent death of the rabbits occurred on the 4th to the 5th day. White mice received Zenkowsky's second vaccine subcutaneously in a dose of 0.01 ccm. General swellings and death ensued in 40 to 42 hours.

d. Procurement of the edematous fluid: The edematous fluid was obtained by sterile motion from the infiltrate of dead animals (those in agony were killed with ether). We avoided damage to the vessels and did not use edematous fluid with visible admixture of blood. The specificity of the animals' death from anthrax was controlled patholoanatomically and also bacteriologically.

Preparation of Undiluted Native Allergen

The obtained edematous liquid was defibrinated and centrifugated under sterile conditions. The clear centrifugate was filtered on membrane filter No. 3 and then

tested for sterility. The obtained filtrate was mixed with formalin, resulting in a concentration of 0.05 percent. The undiluted native allergen thus obtained, designated "anthrax allergen MIEMG" by us, was placed in ampullae under strictest precautions of sterility and stored for further use at a temperature of 10° C as well as at room temperature.

Immunization of Test Animals

In order to test the biological activity of the allergen on guinea pigs, we immunized them by a single application of vaccine STI-1. The animals which survived the infection instilled for the purpose of obtaining edematous fluid (70 percent) also proved to be immune.

Under aseptic conditions, 0.1 ccm, in some cases 0.2 ccm allergen was carefully, intracutaneously injected into the lateral portion of the guinea pig's shorn trunk. The reaction was read in all cases after 24 and 48 hours, in some cases subsequently 6 and 8 hours after the test run. Testing of immunized guinea pigs was started 25 to 120 days after vaccination.

Test Results: The Biological Activity of Anthrax Allergen MIEMG

Anthrax allergen MIEMG from guinea pigs vaccinated with strain STI-1, was applied to 18 immune guinea pigs and 10 non-immune animals. Six hours later the immune animals revealed redness at the stab point (6 animals) or redness with an infiltrate showing indistinct borders and measuring 2 to 10 mm in diameter (12 animals). The cutaneous reaction became more marked, and at the peak of its occurrence (after 24 hours) we observed on all guinea pigs redness at the point of injection with an infiltrate with a diameter of 5 to 36 mm, average size 15-20 mm (13 animals) (Fig. 1). The control guinea pigs did not show a skin reaction at the same observation times. The reaction began to abate approximately 48 hours later and in those cases where 0.2 ccm allergen was administered (5 animals), necrosis developed in the center of the infiltrate (Fig. 2).

The second group, consisting of 4 immune animals and 2 non-immune control guinea pigs, received allergen from guinea pigs infected with Zenkowsky's second vaccine. Within 24 hours redness and an infiltrate with a diameter of 15-35 mm were seen at the point of injection of all immune animals; no reaction occurred on the control animals.

The third group, consisting of 4 immune guinea pigs and 2 non-immune control animals, received rabbit anthrax allergen. After 24 hours, the redness with infiltrate at the stab point measured 17-20 mm in diameter on 3 immune animals. One immune guinea pig and both control animals failed to react.

The fourth series of 3 immune and 2 non-immune guinea pigs received mouse anthrax allergen, diluted 1:1 with physiological saline as required. All immune guinea pigs revealed a corresponding reaction, manifested by redness and infiltration 7-10 mm in diameter at the point of injection, accompanied by a total absence of symptoms in the control animals.

The Biological Activity of Diluted Allergen

Anthrax allergen MIEMG (from guinea pigs infected with STI-1), diluted 1:4 with sterile saline solution as required, was instilled in 4 immune and 3 non-immune guinea pigs. One of the immune guinea pigs showed a small cutaneous reaction at the point of injection after 6 hours, which disappeared approximately 18 hours later. This guinea pig's condition was visibly anergic and the animal died 7 days later from exhaustion and avitaminosis. The remaining 3 immune animals showed, after approximately 24 hours, redness and infiltrate with a diameter of 10 mm at the site of injection. No reaction was seen in the control animals.

Test with Allergen Mixed with Hyperimmune Serum

Allergen from guinea pigs infected with STI-1 was mixed with a two-fold quantity of serum hyperimmune against anthrax. The mixture was allowed to stand for 24 hours at room temperature, then applied to 4 immune guinea pigs in the usual manner. All animals revealed an allergic skin reaction with redness and infiltration reaching a diameter of 5-10 mm after about 24 hours.

Test with Heated Allergen

Allergen from guinea pigs infected with STI-1 was heated on the water bath at [illegible]° C for 30 minutes. It was then instilled in 4 guinea pigs in the usual dose of 0.1 to 0.2 ccm. On all animals an allergic reaction was noted which, approximately 24 hours later, had reached a diameter of 5-12 mm of redness and infiltrate.

Testing Anthrax Allergen MIEMG for Specificity

Nine guinea pigs immune to tuberculosis received anthrax allergen MIEMG emanating from guinea pigs and rabbits, infected with STI-1 as well as Zenkowsky's second vaccine. Three guinea pigs immune to tuberculosis (control animals) each received 0.1 ccm tuberculin intracutaneously. After 24 and 48 hours not one guinea pig inoculated with BCG vaccine reacted to the application of anthrax allergen, while those animals which had received tuberculin showed a distinct cutaneous reaction within about 24 hours, manifesting redness with an infiltrate at the site of injection of 12-22 mm in diameter.

A similar test was conducted with 6 guinea pigs immune to tularemia who received anthrax allergen in the usual manner. Those additional guinea pigs immune to tularemia were given 0.1 ccm tularemia intracutaneously. Five of the six animals immune to tularemia did not react to anthrax allergen, one animal showed a slight redness at the site of application, which disappeared after 30 hours. Of the 3 control animals, two reacted to tularemia with an allergic reaction, one did not react at all (anergy).

The Time in Which the Allergic Reaction Occurs in Guinea Pigs Immunized against Anthrax

Four healthy guinea pigs were immunized against anthrax in the manner described

above. On the fifth day after immunization, they received homologous anthrax allergen MIEMG. All guinea pigs revealed a positive skin reaction (in 24 hours the redness with infiltration assumed a diameter of 5-10 mm).

Positive Anthrax Allergy as an Indicator of Resistance to the Infection

Five immune guinea pigs which had shown a positive cutaneous reaction to anthrax allergen MIEMG, received an intracutaneous injection of 65-75 absolutely lethal doses of anthrax bacilli. The same quantity was given to five non-immune healthy control animals. The control animals succumbed to anthrax within 36-48 hours. The immune ones survived (period of observation 10 days).

Discussion of the Results Achieved

The method suggested by us makes it possible to obtain a specific allergen, the use of which enabled us to determine the phase of injection and immunity to anthrax in guinea pigs. Forty-six of 48 tests (96%) with all forms of allergen (undiluted, diluted, heated, mixed with hypersensitive serum), produced from various anthrax strains and tested on three animal species, gave positive reactions. The gross co-efficient of negative reactions (4%) in one case is due to the doubtlessly anergic condition of the animal. The specificity of the allergen, which in toto evoked one single unspecific and indistinct parallergic reaction in an animal immune to tularemia, was convincingly proved. This circumstance is especially important, since Balteanu et al (1943) upon instillation of a self-made preparation in tuberculosis patients, noted a parallergic reaction in 33.1%. We assume that the impressive parallergic and other skin-irritating properties of Balteanu's preparation are due to the fact that virulent capsulating microbes were used in the production. This assumption is supported by Preiszs, who pointed to the occurrence of substances emanating from capsules (whose injurious effect was admitted by all) in the form of a soluble substance in the edematous fluid and the blood of infected animals.

The data contained in our report again confirm the independence of the immune-biologic effect in the organism from the consideration whether or not the anthrax pathogen has a capsule; the closest approach to reality should be represented by this observation: The less virulent the anthrax strain which causes swellings, the more distinct is its ability to form protective antigen and the allergen related to this effect in the living organism.

The isolation of anthrax allergen from various animal species by means of infection with non-capsular as well as with weakly virulent capsulating anthrax bacilli and the positive results of the crossed employment of these preparations on one hand point to the heterologous character of the allergen, on the other to the fact that it is derived from the soma of the bacillus and not, under any circumstances, from its capsule.

We agree with Houschka in the opinion that the obtained preparation is not identical with Bail's aggressins. The main difference lies in the thermostability

the allergen and the circumstance that it cannot be neutralized with hyperimmune serum. We should like to add that the production of anthrax allergen from non-capsular pathogens (not accomplished by Houschka, nor by Balteanu, et al) by itself excludes an identity with aggressin, which, as is well known, is obtained from the capsular substance.

The isolated anthrax allergen differs by its thermostability from the protective antigen and also from the inflammatory factor discovered by Cromartie and co-workers in 1947, and differs from the lethal toxic factor described by Smith and Keppie (1954) by the absence of the neutralization effect in a mixture with hyperimmune serum.

The severity of the skin reaction is directly proportional to the quantity of applied allergen, according to our experiments. A positive skin reaction following application of the preparation obviously not only indicates an allergic alteration of the tested macroorganism, but also its resistance to a lethal infection with anthrax.

Figure 1. Allergic reaction of the immune guinea pig 24 hours after intracutaneous application of anthrax allergen.

Figure 2. Central cutaneous necrosis formed on the guinea pig at the site of the allergic reaction after 48 hours (enlarged 1.5 X).