IMMUNOLOGIC RELATIONSHIP OF DONOVANIA GRANULOMATIS TO GRANULOMA INGUINALE*

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PLATE 3

(Received for publication, September 5, 1944)

There is very little indication of acquired immunity to granuloma inguinale. Greenblatt (1) refers to a localized infection on the vulva of a white woman that was self-limited, healing without therapy. This is the only instance of the kind that has come to our attention. The characteristic course is one of progressive though gradual extension. Remissions after apparent therapeutic cure are not uncommon and inoculatory lesions, sometimes at a distance from the original site of infection are described. The natural history of the infection then is one of exquisite chronicity.

Initial infections, natural or artificially induced by the injection of Donovan bodies, have not been sufficiently studied in detail to determine a difference between the earliest stages and the commonly seen chronic granulomatous phase that might point to a heightened or modified resistance due to the establishment of some degree of general or local immunity in infections of longer duration.

When one examines microscopically the granulation tissue from an active lesion there are elements in the reaction which led DeMonbreun and Goodpasture (2) to express the opinion that the freeing of Donovan microorganisms from their characteristic intracellular position by rupture or disintegration of the host mononuclear cell liberated simultaneously an injurious material which was responded to by a focal accumulation of polynuclear leucocytes, and that repetition of such minute injuries kept the granulation tissue constantly reforming (4, Fig. 1). The presence of miliary foci of leucocytes in the granulation tissue of the active lesion is a conspicuous feature, and if one assumed the existence of a sensitized state or the development of humoral antibodies to specific antigens of the etiologic agent these little pustules might well be explained on the basis of specific tissue injury or a localization of antigens by serum antibodies. However there existed heretofore no experimental evidence to support such an assumption.

With the discovery by Anderson of a method of cultivating from granuloma inguinale a microorganism in pure strain having characteristics of the Donovan body, it became immediately feasible to undertake studies of the antigenic and

* Aided by a grant from the John and Mary R. Markle Foundation.

immunologic relations between this agent and the human patient (3). Such studies have been in progress for the past several months, and all accumulated evidence substantiates the view that the cultured microorganism in an encapsulated state is the specific "Donovan body" and that the Donovan microorganism is the etiologic agent of granuloma inguinale. Evidences of this are that the cultured agent when inactivated by heat, or its capsular substance separated from the microorganism, will elicit a localized inflammatory response in the skin of patients apparently specific for granuloma inguinale; that inactivated serum from these patients will manifest precipitation and complement fixation responses to the addition of a mucinous, presumably capsular substance, formed by the microorganism under conditions of culture.

It is the purpose of this paper to describe the methods and materials used in these experiments, and to discuss briefly our interpretation of the results.

Antigenic Material Obtained from Cultured Donovan Microorganisms

In an accompanying paper the method used in the cultivation of the presumptive etiological agent of granuloma inguinale from three cases of the disease has been described (4). We have used the microorganism, propagated in series in the yolk of chick embryos, to prepare a "bacterial" antigen; and a sticky, mucinous material, apparently secreted by the growing microorganism in this medium and on a modified solid medium, was purified and used as a presumptive specific capsular substance.

Preparation of the Bacterial Antigen.—Bacterial antigen was prepared from the yolk of embryos infected on the 5th day of incubation with the Donovan microorganism of the 50th generation of strain I and incubated for 4 days at 37°C. A good growth was determined by examination of smears of yolk stained by Wright's method.

Ten cc. of thin yolk from each of 3 live embryos were aspirated with a sterile syringe and needle and pooled. The 30 cc. of yolk were first centrifuged lightly at 40 R.P.M. for 5 minutes to throw down gross particles. The supernatant was aspirated and diluted with two parts of sterile 0.9 per cent NaCl solution and rendered distinctly alkaline to litmus by the addition of 5 per cent Na_2CO_3. The addition of the alkali served to clear the yolk apparently by dissolving suspended material that otherwise would have been thrown into the sediment on centrifugation. The alkali also served to dissolve capsular material.

The diluted alkalinized yolk was centrifuged at 2000 R.P.M. for 1 hour. The supernatant was discarded and the sediment, composed for the most part of bacterial cells together with a small amount of insoluble yolk granules, was resuspended in 100 cc. of 0.9 per cent NaCl solution. The bacterial cells were thus washed twice and separated by centrifugation. The final sediment was suspended in 25 cc. of sterile 0.9 per cent NaCl solution. This served as the stock suspension from which dilutions for the skin tests were made. The suspension was distinctly turbid and contained a recognizable amount of yolk granules in stained smears together with an abundance of bacterial cells. The amount of bacteria present per cubic centimeter was arbitrary and no attempt at a quantitative estimation was made.

The final suspension was immersed in a sealed bottle and heated at 60°C. for 1 hour. Heated antigen inoculated into the yolk sacs of developing embryos 5 days old, and onto blood agar plates, showed no evidence of bacterial growth.

Preparation of "Filtered" Antigen.—A thin portion of infected yolk was centrifuged at 40 R.P.M. for 5 minutes. The supernatant was diluted with an equal volume of 0.9 per cent NaCl solution and centrifuged at 2000 R.P.M. for 1 hour. The supernatant from this centrifugation was filtered with difficulty through a Berkefeld V filter. A very small volume of filtrate was obtained.

A control filtrate was obtained in the same way from uninfected yolk of the same aged embryos.

Preparation of "Capsular" Substance.—Beginning with the 14th generation of strain I it was noticed that the infected yolk of embryos 4 days after inoculation appeared slightly sticky and mucoid. This characteristic increased in subsequent generations so that the yolk became very mucoid and ropy. Stained smears from such yolk indicated that the mucoid substance was the product of the bacterial cell and represented capsular material secreted into the yolk. Globules and strands of pink-staining material became abundant as manifested by smears stained by Wright's method and capsular halos about individual microorganisms were shredded and apparently directly related to the similar dispersed material.

Acting upon the assumption that the mucoid material was capsular in origin an attempt was made to recover it in a purified form for serological tests. For this purpose cultures of the 70th generation of strain I in the yolk of developing embryos were used. Five-day-old embryos were inoculated in the yolk sac and 10 cc. of thin mucoid yolk were removed 4 days later from each of 3 embryos. This was pooled for further treatment.

The 30 cc. of thin yolk were centrifuged lightly at 40 R.P.M. for 5 minutes to remove gross particles. The supernatant was aspirated and diluted to 100 cc. by the addition of 70 cc. of N/100 NaOH. It was then centrifuged at 2000 R.P.M. for 1 hour. The supernatant was removed by aspiration and 10 cc. of N/10 HCl were added. An abundant stringy and sticky precipitate formed which was easily collected on a glass stirring rod and removed almost quantitatively from suspension.

The precipitate was then dissolved in 100 cc. of N/100 NaOH by agitating the adherent precipitate on the rod and rubbing detached strands of it on the side of a 500 cc. Erlenmeyer flask. The precipitate had a yellowish color, and in order to remove coloring matter and adherent lipoid material the dissolved precipitate was extracted with 100 cc. of anesthetic ether. This was accomplished by shaking and extraction in the ice box for 12 hours. The aqueous solution was removed from beneath the overlying ether by aspiration and again extracted in a similar way with one volume of ether.

After removal from the second extraction the aqueous solution was subjected to a negative pressure of 25 pounds by means of a water pump until the ether was removed.

Reprecipitation was accomplished by the addition of an equal volume of N/100 HCl and again the precipitate was collected and removed with a glass stirring rod. It was then washed off the rod into 100 cc. of sterile distilled water and the white flakes and shreds thoroughly washed twice in 100 cc. of distilled water. It was finally removed by centrifugation and resuspended in 30 cc. of N/100 NaOH. This solution had a distinctly opalescent appearance. It was distributed into five 10 cc. vaccine bottles, heated to 60°C. for 1 hour, and cultured in embryonic yolk and on blood agar plates. No bacterial growth appeared. This final solution served as stock for the subsequent immunologic tests.

Immune Reactions in the Skin of Human Patients

Skin Tests with "Bacterial" Antigen.—

Six negroes with active lesions of granuloma inguinale were given skin tests for sensitivity to the bacterial antigen. These patients were first tested for hypersensitivity to yolk proteins by an intradermal injection of 0.1 cc. of 1:1000 dilution of the control filtrate. One-tenth cc.

of the stock bacterial antigen diluted 1:100 with sterile 0.9 per cent NaCl solution was injected intradermally on the anterior surface of the left forearm. On the corresponding surface of the right forearm 0.1 cc. of 1:10 dilution of the test yolk filtrate and 0.1 cc. of 1:10 dilution of the control yolk filtrate were injected intradermally at points separated from each other. The skin reaction reached its height at 24 hours. Each patient reacted positively to the bacterial antigen. Areas of marked erythema with slight elevation ranged in different individuals from 8 cm. by 4 cm. to 11 cm. by 8 cm. There was usually a small area of about 1 to 2 cm. in diameter at the point of injection that was more intensely red and slightly indurated. This reaction was accompanied by mild itching. There was never any local necrosis or ulceration and no constitutional symptoms. The reaction faded fairly rapidly during the next 24 hour interval and was practically gone by the 3rd day.

The test filtrate gave definite but milder reactions ranging in size from 3 × 2.5 cm. to 10 × 6 cm. The control or normal filtrate gave reactions of slight redness 1.5 × 1 cm. or less with one exception. The exceptional patient was most reactive to each injection and the accompanying photograph (Fig. 1) shows the pronounced contrast between her control and test reactions.

One patient with a history of granuloma inguinale that had been healed for 2 years tested in like manner gave so little reaction to either antigen and control that the sites of injection could scarcely be located at 24 hours.

One patient with lymphopathia venereum, a positive Frei test, and no indication of granuloma inguinale, gave a negative reaction to the bacterial and filtered antigens. Another patient with a positive Frei test and lymphopathia venereum gave an evident reaction to the injections but the reaction to the control filtrate of yolk was just as pronounced as the reactions to the materials containing Donovan antigens. This patient was judged to have demonstrated a sensitivity to yolk proteins.

A tenth patient with a penile lesion from which Donovan bodies could not be demonstrated gave a positive reaction to the bacterial antigen. Subsequent circumcision failed to reveal a hidden lesion of granuloma inguinale. There was clinical evidence that this lesion was chancroid.

These experiments, in spite of one apparently false positive reaction, indicate a specific antigenic relationship between the bacterial body of the Donovan microorganism isolated and cultivated in embryonic chick yolk and the cutaneous tissue of patients with active lesions of granuloma inguinale. Positive skin reactions to filtered infected yolk in the same patients indicate the presence in this medium of a specific substance separable from the bacterial body. Negative reactions to the bacterial antigen in 3 patients without granuloma inguinale and negative reactions to normal yolk filtrate support this interpretation. This series of experiments is short but it indicates the potentiality of obtaining a more highly refined and a standardized antigen from a cultured strain of the Donovan microorganism that would be useful in eliciting a specific skin reaction diagnostic of granuloma inguinale. We have not had opportunity to investigate any antigenic relation between this microorganism and patients with proved chancroid.

Skin Tests with "Capsular" Substance.—The specific and positive skin reactions in patients with granuloma inguinale to filtered yolk of infected embryos indicated the possibility of there being in infected yolk a precipitable substance

of a mucoid or capsular nature. The precipitable or "capsular" material was prepared as described above.

Four patients with active lesions of granuloma inguinale were given intradermal injections of 0.1 cc. of 1:5 and 1:10 dilutions of our stock solution. Each patient gave a positive reaction to the 1:5 dilution. The reaction was milder, less easily read, and the area of reaction was about one-third the size of the bacterial test. There was an area of erythema 3 × 2.5 cm. with 1:5 "capsular" antigen in the same patient who gave a 11 × 5 cm. reaction to 1:100 dilution of the bacterial antigen. A 1:10 dilution of capsular antigen in the same patient gave only the slightest redness at the site of injection. Stronger dilutions (1:1 and 1:2) tended to give too noticeable a reaction in control patients to be used as test dilutions. Reactions with these dilutions (1:1 and 1:2) in control patients were of a different quality from the test reaction. They were characterized by less erythema and a more pronounced induration. A total of 17 control tests with the "capsular" material were performed. Nine were tested with 1:5 and 1:10 dilutions and each gave a negative reaction to each dilution. Eight were tested with 1:1 and 1:2 dilutions. Two of the 8 gave negative reactions to both dilutions. Reaction in the other 6 ruled out the use of 1:1 and 1:2 dilutions as useful for the test.

In summary 4 patients with granuloma inguinale gave mild, positive skin reactions to 1:5 dilution of the capsular substance. Nine control patients, one of which was Frei-positive, gave negative reactions to the same dilution of the same substance. This reaction like that to the bacterial antigen was best read at 24 hours.

The comment on the usefulness of the various dilutions of this "capsular" substance has meaning only with reference to the concentration of precipitated substance present in our stock solution. A precise procedure for preparing and standardizing this substance has not been established but a specific nature of this precipitable material in infected yolk is indicated. From these short series with single particular lots of bacterial antigen and "capsular" substance it appears that the bacterial antigen elicited the best skin reaction. We have not, however, tested for confusing reactions that this antigen might give with antibodies to other Gram-negative bacilli. We also recognize that a more refined capsular substance might give a better specific reaction in greater concentration without reaction in controls.

Specific Antibody Reactions in Vitro

The apparently specific immune reactions elicited in the skin of patients with granuloma inguinale by the intradermal injection of "capsular" substance precipitated from embryonic chick yolk infected with the Donovan microorganism indicated the possibility of demonstrating the presence of specific humoral antibodies in patient's serum by precipitation and complement fixation tests *in vitro*. All the experiments were done with a single stock solution of "capsular" substance and this was the same lot used for the skin tests.

Tests for Specific Precipitation Reaction.—Three types of phenomena involving a precipitation reaction were observed, namely, ring formation, flocculation, and sedimentation.

Preliminary experiments showed that undiluted serum with an equal volume of a 1:10 dilution of the particular lot of "capsular" substance gave the best precipitation. Test tubes 2¼ inches long were made by sealing one end of glass tubing having a 3 mm. internal diameter. Materials were introduced with capillary pipettes. Five-hundredth to 0.07 cc. of serum was pipetted into the bottom of the tube and a column of 1:10 dilution of the "capsular" substance of equal height was layered above the serum. Often a ring of precipitate formed at the interface almost immediately. Final examination for the formation of a ring was made after 2 to 3 hours' incubation at 37°C. The serum and specific substance were then mixed by inversion and rotation of each tube. Further incubation at 37°C. for 5 to 6 hours was allowed before recording the presence or absence of gross or microscopic (×10 ocular lens) flakes of precipitated particles. The tubes were allowed to stand overnight in a refrigerator at about 5°C. and an observation upon sedimentation of the precipitated particles was made.

Human serum from 86 individuals was tested for precipitation reactions as described above. Serum was inactivated in a water bath at 56–57°C. for 30 minutes. This inactivation was necessary because of a non-specific precipitation elicited by the use of fresh serum. With the exception of one serum that had merthiolate added no preservative was used.

Of the 86 serums 19 were from patients with lesions of granuloma inguinale diagnosed by the presence of Donovan bodies in smears. Eighteen of the 19 gave positive ring, flocculation, and sedimentation tests. One of these reactions may be described as weak. It was on a sample of serum over a year old with a precipitate that required removal by centrifugation. This patient had died and fresh serum was not available. Other old serums without precipitates gave stronger positive tests. A twentieth serum, which gave a weak precipitating reaction, was from a patient whose infection had been of only 9 months' duration and whose lesions were already healed as a result of chemotherapy before the serum was taken. The one serum from a patient with granuloma inguinale that failed to give a positive precipitation reaction was from a patient whose infection was of only 7 months' duration and whose lesions were improving under treatment but not completely healed. Ten of the 19 serums gave strong ring tests, abundant course flocculations, and heavy fluffy sedimentations. The other seven serums were described as giving a moderately strong ring test, moderate and fine flocculation, and moderate sedimentation.

The remaining 66 serums comprising the control group from patients without any history or evidence of granuloma inguinale can be divided into particular control groups. Serum from 6 patients with Frei-positive skin reactions and with clinical histories of lymphopathia venereum were tested. Five gave negative precipitation tests. One gave a moderate ring test and flocculation without sedimentation. Serum from 15 hospitalized patients with positive Wassermann and Kahn tests reacted negatively to the granuloma inguinale material. Serums from 26 patients in the hospital that gave negative Kahn tests were tested for precipitation by the "capsular" substance. Twenty-five were negative and one was positive. This one patient was in the hospital for treatment of active pulmonary tuberculosis. There was no history or evidence of granuloma inguinale. One serum from a patient with a very early chancroid infection gave no precipitation. The remaining 18 control serums were from normal healthy individuals who were donating blood to the Red Cross blood bank. They gave negative Kahn reactions and were negative when tested for precipitation with the "capsular" substance.

To summarize, 19 of 20 granuloma inguinale patients' serums gave positive precipitation reactions and of 66 control serums all were negative except 2.

Tests for Complement Fixation.—

Complement fixation tests were carried out on 17 of the granuloma inguinale serums and on 20 of the control serums used for the precipitation tests. Complement, sheep cell amboceptor,

and 2 per cent sheep red blood cells were standardized as for use in the Wassermann laboratory at Vanderbilt Hospital. Preliminary tests indicated that undiluted patient's serum and 1:10 dilution of the "capsular" substance established the best combination. One-tenth cc. human serum, 0.1 cc. 1:10 stock "capsular" substance, and 0.5 cc. complement were mixed and raised to a volume of 1 cc. with 0.9 per cent NaCl solution. This mixture stood in an ice box overnight and then after 10 minutes' incubation in a water bath at 37°C., 0.1 cc. amboceptor, 0.2 cc. sheep red blood cells, and saline to a volume of 1 cc. were added. Incubation in a water bath at 37°C. for 30 minutes carried the test to completion. A second reading on sedimentation of red cells after standing in the ice box overnight was made. Each serum was tested for an anticomplementary effect. The "capsular" substance did not fix complement.

Of the 17 granuloma inguinale serums tested 2 were anticomplementary, 6 fixed complement completely, 6 showed partial fixation, and 3 apparently failed to fix complement.

Of 20 control serums 1 was anticomplementary, 1 showed the very slightest amount of fixation, and 18 failed to demonstrate fixation.

In summary, of 15 non-anticomplementary granuloma inguinale patients' serums, 12 fixed complement either partially or completely and 3 failed to demonstrate fixation. Of 19 non-anticomplementary control serums 18 fixed no complement and 1 serum appeared to fix a very slight amount.

Although the number of serums tested in this preliminary experiment was small, the results are believed to demonstrate that a significant proportion of serums from patients with active lesions of granuloma inguinale will fix complement in the presence of a substance which we believe to be capsular in origin, prepared as we have described from yolk infected with the Donovan microorganism after its adaptation to this medium by serial culture. These experiments support the etiologic significance that we attach to this microorganism.

The precipitation and complement fixation reactions did not always parallel each other in a single serum. For example the 3 patients' serums that appeared not to fix complement gave definite precipitating reactions. The 2 serums described as weakly precipitating serums demonstrated partial rather than complete fixation of complement. Unfortunately no serum was available for complement fixation from the single patient whose serum gave a negative precipitation. On the other hand 1 control serum that gave a ring test failed completely to fix complement. We did not attempt to titrate serums that showed partial fixation. From available histories there appeared to be some relation between the duration of the disease, the progress toward healing, and the titer or strength of immune substance in the patient's serum.

Immunization of Chickens

Aside from any etiologic aspect, it seemed of some value, in the light of the above described serological reactions, to demonstrate experimentally the antigenic potentiality of the microorganism that we had isolated from lesions of granuloma inguinale and from which we had obtained an apparently specific "capsular" substance.

To obviate the complication of immune bodies to chick yolk proteins, adult chickens were used as the test animal. Normal serum was collected from the blood of 2 adult Plymouth

Rock roosters. The roosters were subjected to 2 series of intravenous injections of 2 cc. of heavily infected yolk diluted 50 per cent with saline to make it less viscous. After a total of 12 injections the roosters were bled and serum collected. Normal serum gave negative precipitation reactions and immune serum gave positive ring, flocculation, and sedimentation tests with the "capsular" substance. When tested for complement fixation the normal chicken serum was anticomplementary. The immunized roosters gave positive skin reactions to intradermally injected stock bacterial antigen. At the 24 hour interval this reaction appeared as a large edematous area that was pale rather than hyperemic. The edema was strikingly absent from non-immunized chickens.

This experiment would seem to establish the inherent antigenic potentiality of this cultivated strain of the Donovan microorganism and to make tenable the hypothesis of the presence of an antibody in human granuloma inguinale patients homologous with this microbial antigen.

It is worthy of note that although the roosters used for the above immunization experiment were injected with heavy cultures of viable microorganisms no evidence of pathogenicity was observed either locally or generally.

DISCUSSION

Two objectives were in our minds in carrying out the experiments which we record relative to specific sensitization and antibody reaction in the skin and serum of patients with granuloma inguinale. One of these objectives was to acquire, if possible, further evidence of an etiologic relationship of the microorganism cultivated from 3 cases of the disease with active lesions. The second objective was to determine whether or not from a practical standpoint a skin test or a specific serological test might offer possibilities of usefulness as a diagnostic aid.

Morphological studies of the microorganism cultivated in embryonic yolk inoculated with uncontaminated material from human lesions revealed so exactly the appearances of the microorganism observed in smears from human lesions that we were satisfied on formal grounds that we were dealing with the agent identified by every recent student of the disease as constituting a pathognomonic feature of the lesion of granuloma inguinale, namely, the Donovan body. We were further confirmed in this impression by the fact that the cultivated microorganism required for its reproduction the special conditions which exist in the yolk of living chick embryos, because numerous competent investigators have arrived at the conclusion that the Donovan microorganism will not grow on any of the artificial bacterial media in common use (5, 6).

The skin reactions with a bacterial antigen and the serum reactions with a material derived from infected chick yolk, which we believe to be capsular in origin, are confirmatory in our minds of the judgment arrived at on morphological and cultural grounds, that the microorganism with which we are dealing is the etiologic agent of granuloma inguinale. In an accompanying paper we have presented reasons for regarding this microorganism as a bacterium and have

proposed, because of its peculiarities, the creation of a new genus *Donovania*, species, *granulomatis*, to give it taxonomic status (4).

In regard to the possibility of the development of specific diagnostic tests, such as a skin test, precipitation, and complement fixation tests, on the basis of the preliminary immunological reactions which we have described, we feel that the conditions are very favorable. In this particular study we have not made an attempt to refine the methods or to apply them to the number of patients with this and other diseases to warrant a categorical statement as to the merits of such tests but we believe the indications deserve further and more intensive study in this direction. Much remains to be done with reference to the purification and analysis of the antigens and specific substances which are responsible for the reactions we have observed. The bacillary suspension which constituted our bacterial antigen was not entirely free from organic material from the yolk. The control tests used for the skin reaction definitely ruled out a participation of yolk protein in the amounts remaining with this antigen in inducing the reactions observed.

We have not attempted a chemical analysis of what we call the "capsular" substance although its mucinous character and its apparent morphological relationship to the capsule of the Donovan microorganism, and its content of reducing substances incline us to the opinion that it is largely carbohydrate in composition. We have made no observation as to whether or not this precipitated "capsular" material is antigenic.

SUMMARY

1. A washed bacterial suspension of Donovan microorganisms cultivated in the yolk of chick embryos was used as an antigen in intracutaneous tests in 6 cases of granuloma inguinale in the active stage of the disease. These injections were responded to by an extensive erythematous and edematous reaction that reached its height in 24 hours and disappeared within 48 hours later. Four control cases without granuloma inguinale did not react to the injection to any greater extent than would be expected from the slight trauma incident to them. The skin of the reacting patients responded in a similar manner but to a lesser extent to a filtrate of infected yolk. Simultaneous control injections of filtered normal yolk demonstrated that sensitivity to normal yolk was not responsible for the reactions.

2. A mucoid material present in infected yolk, soluble in N/100 NaOH, and precipitable by N/100 HCl was recovered and purified by repeated acid precipitation, extraction with ether, washing in distilled water, and dissolution in N/100 NaOH. We regard this material to be of capsular origin.

3. The presumptive capsular material injected intradermally elicited a somewhat different and milder reaction in patients with active granuloma inguinale, but we regard it as specific.

4. The "capsular" material in suitable dilution elicited distinct precipitation reactions when mixed with the serum from 18 of 19 cases of active granuloma inguinale. Serum from 1 active case showed no precipitation. Sixty-four serums, including 6 from lymphopathia venereum patients and 18 from Wassermann- or Kahn-positive patients, and 1 from an early chancroid failed to precipitate. Two serums from a total of 66 used as controls showed a precipitate in the presence of the capsular material. These patients gave no history or evidence of granuloma inguinale.

5. The "capsular" material fixed complement in the presence of serum from patients with granuloma inguinale. Serum from 12 of 15 cases of granuloma inguinale demonstrated complement fixation. Three failed to fix complement. Of 19 control serums 18 failed to fix complement in the presence of the capsular material and 1 fixed only a very slight amount of complement. None of these serums nor the capsular material itself was anticomplementary.

6. The high proportion of precipitation and complement fixation reactions obtained from patients with active granuloma inguinale is indicative of a specific immunological relationship of the Donovan microorganism cultivated in embryonic yolk to the disease, granuloma inguinale.

7. We suggest that one or all of these methods might be so standardized as to be of distinct diagnostic value.

We acknowledge with appreciation the assistance of Dr. Stewart H. Auerbach, University of Georgia, Dr. Gurney Clark, Johns Hopkins University, and Dr. R. H. Rigdon, University of Arkansas, in obtaining serums for this study.

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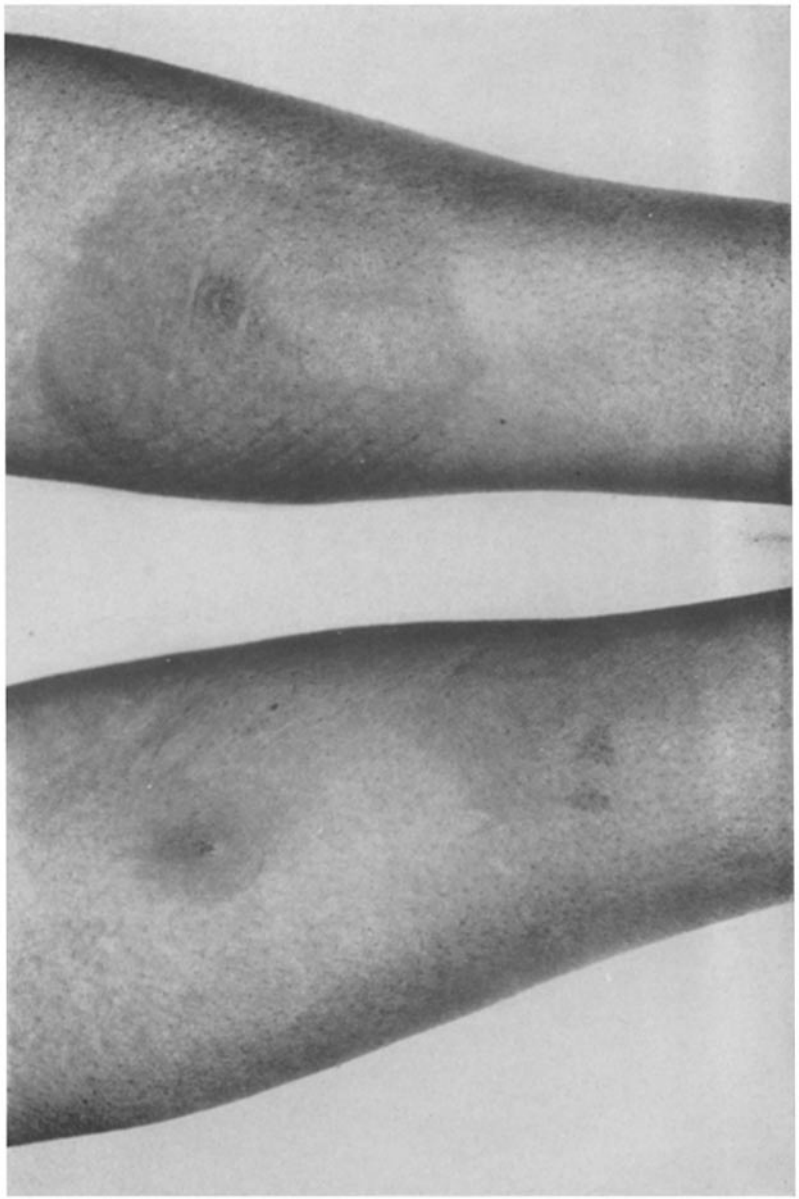
EXPLANATION OF PLATE 3

FIG. 1. Cutaneous reaction in the arm of a negro woman with active granuloma inguinale. Left arm: swelling and erythema over an area 11 × 8 × 1.5 cm. in response to the bacterial antigen. Lower right: area of reaction to filtered infected yolk, 8 × 5 cm. Upper right: reaction to normal yolk filtrate 3.5 × 2.5 cm. 24 hours.



(Anderson *et al.*: Immunologic relation of *D. granulomatis* to granuloma)