6-1970

# Immune Blocking of Fluorescent Antibody Staining of Streptococcus Group A Compared with Anti-streptolysin O

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# IMMUNE BLOCKING OF FLUORESCENT ANTIBODY STAINING OF STREPTOCOCCUS GROUP A COMPARED WITH ANTI-STREPTOLYSIN O

by

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A thesis in Partial Fulfillment

of the Requirements for the Degree

Master of Science in the Field of Microbiology

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June 1970

Each person whose signature appears below certifies that he has read this thesis and that in his opinion it is adequate, in scope and quality, as a thesis for the degree Master of Science.

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Dedicated to my Wife

Muriel

and to our children

Mary and Greg.

ACKNOWLEDGMENTS

The author wishes to express his sincere thanks to the following individuals for their help:

Dr. Charles E. Winter, Chairman of the Guidance Committee, for his patient guidance throughout this investigation and for acquiring the necessary equipment and supplies required for this study.

Dr. Raymond E. Ryckman, for his helpful suggestions in preparing this manuscript.

Dr. U. D. Register, for his helpful suggestions in preparing this manuscript.

Dr. William P. Thompson, pathologist, for clinical specimens used in the early part of the study.

Miss Joyce Ramsey, for assistance in the laboratory.

Others, too numerous to mention here, who aided with suggestions and moral support.

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# INTRODUCTION

## Statement of the Problem

Conjugated Fluorescent antibody methods have been in use for the past twenty-seven years since Coons, Creech, Jones and Berliner located pneumococcal polysaccharide in mouse tissue by using this method in 1942. It was not until some time later that the fluorescent antibody method was applied to the clinical diagnostic field. Goldman (1953) used labeled antibody for the differentiation of Entamoeba histolytica and Entamoeba coli. Little attention was given to the identification of bacterial antigen until 1956. At this time Moody and his associates gave their report using labeled antibody in a series of research projects directed toward the diagnostic identification of bacteria at the public health laboratory level.

The staining of Streptococcus pyogenes with fluorescent antibody was first reported by Moody, Ellis and Updyke in 1958. Group A fluorescent antiserum was tested against various strains of Lancefield's groups A, B, C, D, F, and G. There was some cross reaction with C and less with G. Group A cells treated with untagged homologous antiserum prevented the staining of these cells with fluorescent tagged homologous antiserum. This specific blocking is evidence of the specificity of the fluorescent antiserum. Normal untagged serum failed to do this. These tests were all conducted on streptococcal cells from cultures.

Moody (1958) used the above method in testing for the presence of Streptococcus pyogenes Group A cells in the direct smears from

throat swabs of eight patients with severe sore throats. Four of the smears showed at least 2+ fluorescence of streptococcal cells. (See Materials and Methods, section A - Degree of Fluorescence). Parallel cultures of these same swabs proved to be beta hemolytic streptococcus Lancefield group A. The remaining four swabs did not culture out group A streptococcus. Cherry, Moody and associates in their monograph on fluorescent antibody methods in 1960 stated that staining of group A streptococci in smears made directly from throat swabs was not very successful. In the rare instances where positive reactions were obtained, the fluorescence was of low intensity and the cells were few in number. It was suggested that these organisms may have been coated with an antibody or other substance present in the throat which inhibited staining but not viability.

Roberts and Sherris (1965) demonstrated this blocking ability to be present in approximately 60% of 96 patients serum tested. This blocking was removed completely if the serum was treated with N/10 sodium hydroxide. In another study (1967) they showed 81% of 70 patients tested to have this blocking substance in their serum. According to age groups, those showing the least blocking effect in their sera were the 2-month to 3-year-olds (40%) whereas those showing the greatest blocking were the 21 to 60-year-olds (100%).

Purpose of the Study

The purpose of this investigation was to find if this blocking effect might be caused by or correlated with the antistreptolysin O antibody.

Todd (1932) suggested that a rise in titer of antistreptolysin in serum may be evidence of disease associated with infection by hemolytic streptococci. It was also hoped that this test would be an aid in the study of rheumatic fever. Later (1934) he discovered that there were two kinds of streptolysin: (i) an oxygen-labile form which is neutralized by antistreptolysin; (ii) an oxygen-stable form which is not neutralized by antistreptolysin. In a later paper (1938) Todd referred to these substances as streptolysin O (oxygen-labile) and streptolysin S (oxygen-stable). The "S" form seems to be very weakly antigenic and is of such low titer in normal or infected serum that it does not interfer with the determination of the Antistreptolysin O Titers. The antistreptolysin S titers either remained constant or showed a tendency to fall during and following a beta hemolytic streptococcal infection.

The rise in titer of the antistreptolysin O antibody has been the diagnostic-test of choice for the past thirty-some years in the diagnosis of rheumatic fever and other streptococcal infection sequela. Though the test is widely used, there have been reported a certain percentage of false positive results. Packalen (1948) describes four different causes for non-specific elevations of the antistreptolysin O titer (ASOT): (1) in sera (and pleural exudated) contaminated with certain bacteria, (2) in sera treated with acid

or alkali, (3) in most hepatitis sera, or (4) in pleural exudates of patients with prolonged pleural effusion. Lipid extraction of sera and exudates abolished nonspecific ASOT reaction. The mechanism that seemed to cause this elevation in ASOT was the presence of cholesterol molecules with free hydroxy groups which in turn neutralized the streptolysin.

The measurement of the fluorescent antibody blocking titer (FABT) does not rely upon neutralizing streptolysin and therefore would not involve the afore-mentioned problem of false positives. Nichols (1962) measured the FABT by use of fluorometer.

The scope of this research was not only to measure the FABT but also to compare it with the ASOT. Roberts (1967) measured the fluorescent antibody blocking in sera of a group of patients with ASOT of 500 or more and another group with ASOT's of 50 or less. "There was a tendency toward high titers of blocking substance in those with higher antistreptolysin O titers, but individual sera showed discrepancies." In our laboratory similar studies were attempted on sera of hospital patients who had already had the ASOT's determined. Some of the problems encountered were discrepancies in: (1) age of serum, (2) storage of serum, and (3) not enough high titered sera.

It was finally decided that a more controlled experiment would be desireable using rabbits immunized with different Streptococcus pyogenes group A antigens. ASOT and FABT tests were run on each rabbit serum specimen collected. The ASOT's were run in the usual manner and the FABT was measured by using the slide inhibition test.

## MATERIALS AND METHODS

### A. Production of Group A Fluorescent Tagged Antiserum.

In general, the method used in the production of this antiserum was that recommended by Smith (1965). However as Smith's method was not followed precisely, the procedure will be given in detail here with the deviations.

Besides the specific antigen Streptococcus pyogenes group A, there were absorbing antigens and antibodies also used to enhance the specificity.

Organisms used. Strains of three beta-hemolytic streptococci, belonging to three Lancefield groups, were secured from National Institute of Allergy and Infectious Diseases, U.S. Public Health Service; #61X101 (group A), #26RP66 (group C), and #56X227 (group G). One strain of Staphylococcus aureus was obtained locally, Loma Linda University #134. Immunizing antigens were prepared by inoculating 250 ml of Todd-Hewitt broth with each organism and incubating at 37° C. for 24 hours. The growth from each culture, after centrifugation, was resuspended in 50 ml. of 0.4% formalized saline in a vaccine bottle, left at room temperature for 24 hours and then refrigerated. Each antigen was tested for viabile cells at the end of three days. (In the hands of this researcher, complete killing was not obtained by heating the antigen in a water bath at 56° C. for 45 minutes as done by Smith (1965). The antigens were concentrated or diluted with formalized saline to give a 1.0 - 1.5 optical-density using a Cortex rectangular cell with the Beckman DU spectrophotometer at a wavelength of 540 mu.

Production of antisera. Antisera were produced by immunizing white, 7 pound, New Zealand rabbits. For Staphylococcus aureus, each rabbit was immunized over a 3-week period, with daily intravenous injections for the first 5 days of each week followed by a 2-day rest period. Dosage consisted of 0.5 ml the first day, 1 ml per day for the remainder of the first week, and finally, 2 ml per day for the next 10 injections.

Rabbits injected with streptococci on this protocol did not survive, so those rabbits receiving these organisms were immunized over a 15 day period, using dosages consisting of 0.5 ml per rabbit per day for the first four days, 1 ml per day per rabbit for the next nine days of injections, finally, 2 ml per day each for the last 2 days of injections. Rabbits were test-bled on the seventh day after the last injection. Those found to have a good precipitin titer were bled out the same day and the serum was collected aseptically and stored in vaccine bottles under refrigeration.

Buffered salines:

1. Preparation of carbonate buffered saline (Hyland Laboratories)

   10.0 ml normal saline

   3.0 ml $CO_3$-$HCO_3$ buffer (0.5 M pH 9.0)

   Cherry, (1960)

   2.0 ml acetone

2. Preparation of phosphate buffered saline, N.C.D.C. manual No. 860 (1965)

   a. Concentrated Stock Solution:

   $Na_2HPO4$ (anhydrous; reagent grade) 12.0 grams.

$NaH_2PO_4.H_2O$ (reagent grade) 2.2 grams

NaCl (reagent grade) 85.0 grams

Distilled water to make final vol. 1000 mls.

b. Working Solution (pH 7.5; 0.01M buffer; 0.85% NaCl):

Concentrated Stock Solution 100 mls.

Distilled water to make final vol. 1000 mls.

3. Buffered Glycerol Saline Mounting Fluid, Difco Laboratories, Detroit.

Conjugation of group A antiserum.

Eighty mls. of group A antiserum from one rabbit was obtained. This serum gave a 3+ precipitin test, Lancefield (1938), within five minutes with Streptococcus pyogenes group A antigen extract. The same serum gave no precipitin reaction with Staphylococcus aureus and Streptococcus pyogenes group C antigen extracts and only a 1+ reaction with group G streptococcal extract. Ten mls. of the group A antiserum was absorbed with 1 ml. each of groups C and G cells in a 37° C. water bath for 30 minutes. The supernatant, after centrifugation, was put through a .45 u. "Millipore" filter into a sterile vaccine bottle. Merthiolate was added to the serum as a preservative to a final dilution of 1:10,000.

To test the specificity and blocking effect of the preserved antiserum one drop was added to a smear of group A cells for 45 minutes at 37°, washed with phosphate buffered saline, followed by adding commercial (Sylvana) group A Fluorescent antiserum for 30 minutes at 37° C. After washing and mounting, these cells showed

only a trace of fluorescence.

Total protein, using the biuret method, was found to be 6.2 grams %. After chilling 9.3 mls of the carbonate buffer pH 9.0 to 0° C., 5.0 mls. of chilled serum (310 mgs. protein) were added. To this mixture was added 15.5 mgs. of dry fluorescein isothiocyanate (0.05 mg/mg protein). The mixture was stirred in the refrigerator over night using a magnetic stirrer. The conjugated serum was separated from the non-conjugated stain by use of a Sephadex G25 column, Golstein (1961). This method gave a wide visable band of separation between the conjugated and the unconjugated flourescein stain. To each milliter of conjugate was added 0.05 ml of each C and G antiserum. For the purpose of blocking non-specific stain, the tagged antiserum was diluted 1:5 and 1:10 portions with phosphate buffered saline (PBS) and 0.05 ml of staphylococcal antiserum was added to each ml. of this diluted conjugate. Merthiolate was added to the final product to a dilution of 1:10,000, separated into aliquots for future use and placed in the freezer.

When used, this fluorescent tagged antiserum was diluted so as to produce a 4+ fluorescence with Streptococcus pyogenes group A cells and no fluorescence with group C or G or Staphylococcus aureus cells. This final dilution was found to be 1:90 at first and lowered with age.

Degree of Fluorescence - Intensity of Stained Cells

Degree of intensity of fluorescence was in accordance with the procedure recommended by the National Communicable Disease Center training course on "FLUORESCENT ANTIBODY TECHNIQUES APPLIED TO

STREPTOCOCCUS GROUPING AND OTHER BACTERIAL IDENTIFICATIONS", as follows:

Four plus = Maximal fluorescence, brilliant yellow-green; clear-cut cell outline; sharply defined non-staining center of cell.

Three plus = Less brilliant, yellow-green fluorescent; clear-cut cell outline; sharply defined non-staining center of cell.

Two plus = Less brilliant, but definite fluorescence; less clear-cut cell outline; non-staining center area fuzzy.

One plus = Definite fluorescence, but very subdued, peripheral and center staining of same intensity.

Photomicrographs were taken of stained smears in an attempt to demonstrate the above grades of fluorescence. It was extremely difficult to get all grades of fluorescence in color due to the fact that brightly fluorescent organisms being irradiated with ultra-violet rays lose more than 60% of their fluorescence during the first 15 seconds and more than 80% by the end of one minute, Goldman (1968).

By using the fastest color film available, "one plus" and "two plus" prints were obtained, (figures 1 and 2). These prints were made using a Kodak High Speed Ektachrome Daylight Color film with an ASA rating of 160 pushed to 400 in developing. All pictures were taken with a 35 mm Leica camera with a Leitz micro camera attachment on an American Optical (AO) microscope with an AO

Fluorolume illuminator.

Black and white prints equivalent to one, two, three and four plus fluorescence were obtained by using Kodak Tri-X Pan black and white film with an ASA rating of 400, (figures 3, 4, 5 and 6).

It may be note-worthy here to mention that the National Communicable Disease Center did not demonstrate their grading of fluorescence with color photography illustrations nor have I found any such illustrations in the literature.

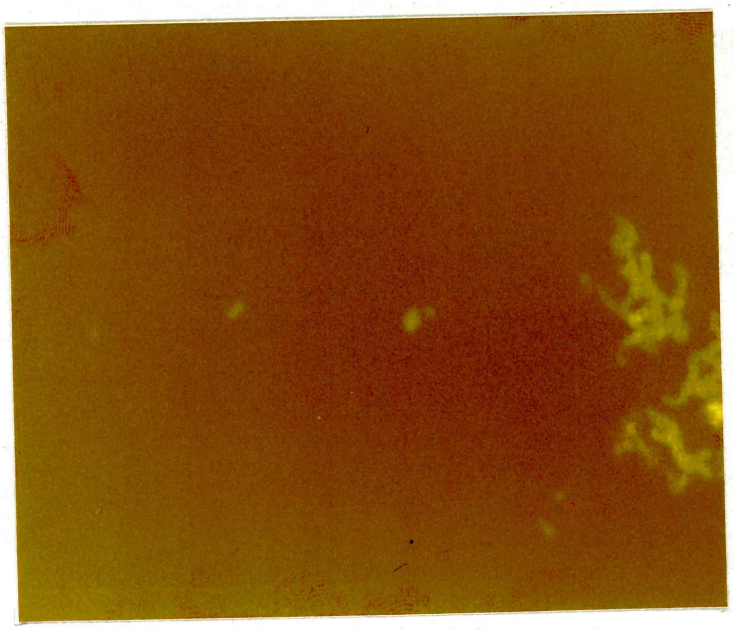

Figure 1. Color photomicrograph of "one plus" fluorescent group A streptococci.

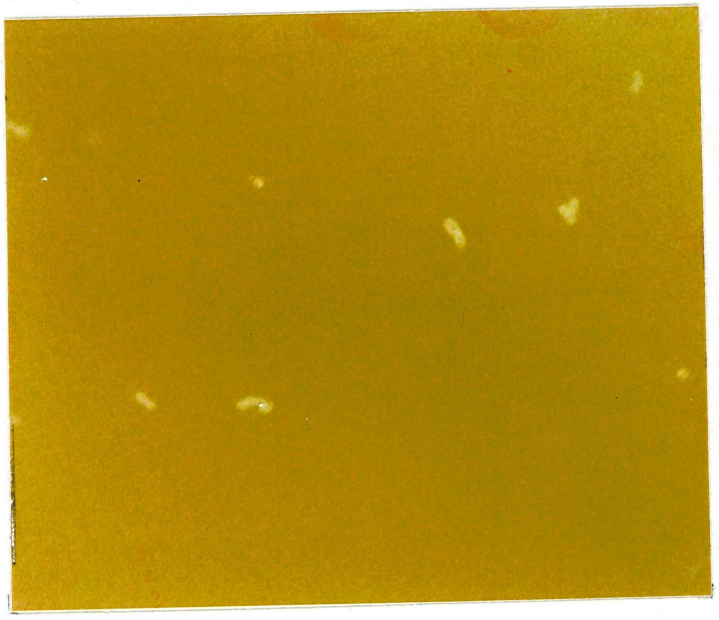

Figure 2. Color photomicrograph of "two plus" fluorescent group A streptococci.

Fig. 3. One plus fluorescence.

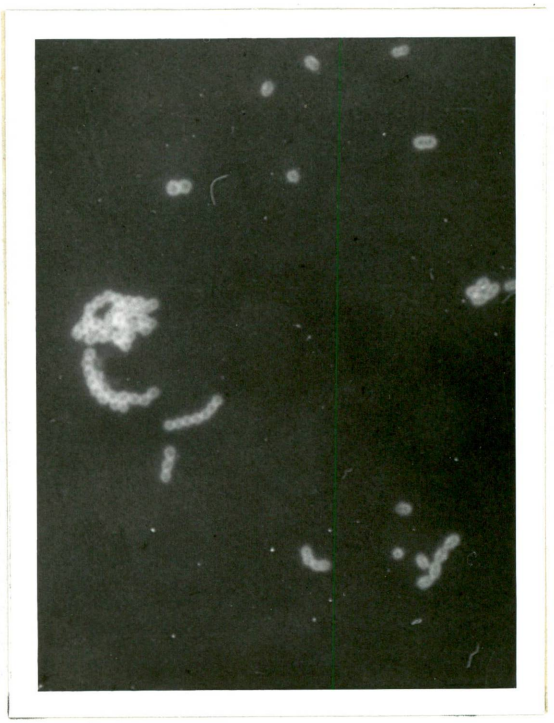

Fig. 4. Two plus fluorescence.

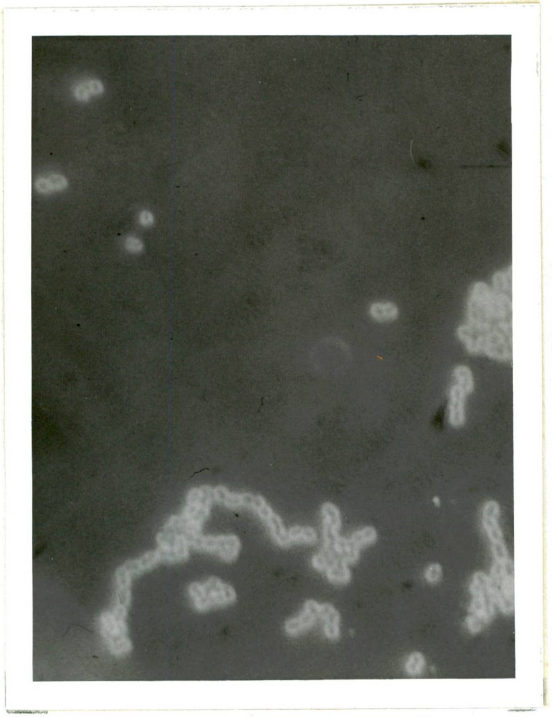

Fig. 5. Three plus fluorescence.

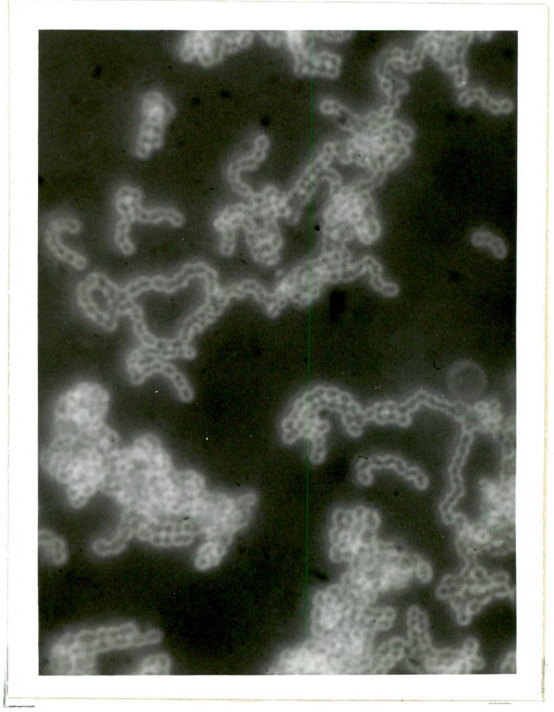

Fig. 6. Four plus fluorescence.

B. Immune Blocking of Fluorescence compared with Streptolysin O Antibody in Human Sera.

As a preliminary part of this study, sera from 100 patients were tested for immune blocking of fluorescent antibody (FAB) against group A streptococcus. These sera were obtained from four different clinical laboratories located in this area. Each specimen, as received from the clinical laboratory, had a label with name of patient, date of collection and titer of antistreptolysin O as determined by the hospital clinical laboratory.

Fluorescent antibody blocking test. A slide inhibition test, Goldman (1957), was used to demonstrate the blocking effect of antibodies occuring in the patient's sera. The antigen used in the slide test were group A streptococcal cells prepared in the following manner:

1. Two to six hour Todd-Hewitt broth culture of group A streptococcus, Moody (1963),
2. Centrifuge 10 ml portions for 5 minutes at 840 G.
3. Decant broth, add 10 ml phosphate buffered saline (PBS) to sediment, mix and recentrifuge as in step 2,
4. Decant PBS,
5. Add PBS to sediment until optical density at 540 mu. wave length reaches 0.75-0.77, using a 16 x 100 mm Kimax #45066 Culture Tube. (This concentration gives good distribution of cells when smears are made.)
6. By use of a capillary pipette, dispense one drop of this sediment to each end of a microscope slide (Curtin #

V58958) and let air dry,

7. Cover each smear with 95% ethanol, keep wet for 1 minute, then let ethanol evaporate. After smears are thoroughly dry, they may be stained or may be frozen and stored and stained at a later date if absolutely no thawing occurs in the interim. Moody (1963).

Actual test for presence of fluorescent antibody blocking (FAB) was performed in the following manner:

1. For the purpose of a double blind test, another laboratory worker removed the patient's serum from the freezer, where it had been stored, thawed it and placed it in another numbered tube to be run. (Not until after the test had been run did the investigator know the identity of the serum.)
2. Remove frozen streptococcal group A slides from freezer and let thaw 20 minutes before using.
3. Add one drop of test serum to each end of slide (2 smears) and incubate 30 minutes at room temperature in moist chamber. A Petri dish fitted with moist filter paper makes a suitable chamber.
4. Shake excess serum onto disinfectant-soaked paper towel.
5. Dip slides momentarily into buffered saline pH 7.5 (PBS) in a staining dish.
6. Transfer to a second vessel of PBS, and let stand for 10 minutes.
7. Dip momentarily into distilled water to remove excess PBS.

8. Blot slides gently with bibulous paper. Never use same area of bibulous paper for blotting more than one smear.
9. Put one drop anti Streptococcus group A (FA) on "S" smear of slide and one drop of anti Human Globulin (FA) on "G" smear of slide, and incubate 30 minutes at room temperature in moist chamber.
10. Repeat step 4 through 8.
11. Add a drop of buffered glycerol saline mounting fluid and a # 1 - 22 mm square cover slip to each smear. Apply one drop of nail polish to each coverslip corner to hold it in place.
12. Stained and mounted smears may be refrigerated and examined anytime within 24 - 48 hours without significant loss of brilliance of fluorescence. Slides to be stored in the refrigerator overnight or longer should be sealed completely with nail polish. CDC (1965)
13. This investigator did not store any of the stained slides overnight but examined the slides the same day using an American Optical Spencer monocular microscope with an AO fluorolume Illuminator equipped with an Osram 200 watt mercury vapor lamp for the light source. An exciter filter (S-BG12/2 mm.)* was placed between the light source and the stained specimen to cut out all light rays except the ultraviolet through blue range. Upon excitation, the

* Code numbers for filters manufactured by Schott and Genossen, Mainz, West Germany.

fluorescent tagged antibody transmitted a clearly visable yellow-green color. A barrier filter (S-OG1/2 mm.) was placed between the stained specimen and the eye ocular lens to absorb any unabsorbed dangerous ultraviolet and near ultraviolet rays.

14. Results were recorded as zero through 4+ as discribed previously under "Degree of Fluorescence". Patients sera numbers 36 - 55 were diluted through a series up to 1:5000 as indicated in table 1 to determine the titer of FAB.

15. A negative control consisted of replacing the patient's serum with PBS. In this control the S smear of the slide should show a 4+ fluorescence while the G smear showed zero fluorescence. A positive blocking test would have the opposite results.

As a further study of the effect of 0.1N NaOH on the blocking antibody, (Roberts and Sherris 1965) a third smear of streptococcal group A cells was treated with the patient's serum as in steps 1 through 8 above. It was then washed momentarily with 0.1 N NaOH followed by staining with fluorescent antibody Streptococcus group A. This was done on the first 50 patient's sera only. The results were always a 3 or 4+ fluorescence indicating that the NaOH destroyed the blocking effect.

Antistreptolysin O titers were verified on all sera. The method used was that of Rantz and Randall (1945) using the dilution scheme in Table 2.

A comparison of antistreptolysin O (ASO) and FAB was done on 18 of the patient's sera.

TABLE 1

FLUORESCENT ANTIBODY BLOCKING TITRATION

| Tube No. | Serum Dilution | Diluted Serum MLS | Fluorescent Antibody Buffer, MLS | Diluted Serum on slide Amount | INCUBATION | Standardized FA Antistrep. on slide Amount | INCUBATION | Titer in FAB Units | Tube No. |
|---|---|---|---|---|---|---|---|---|---|
| 1 | undiluted | 1.0 | 0.0 | 1 drop | | 1 drop | | 0 | 1 |
| 2 | 1:10 | 1.0 | 0.0 | 1 drop | | 1 drop | | 10 | 2 |
| 3 | 1:10 | 1.0 | 1.0 | 1 drop | | 1 drop | | 20 | 3 |
| 4 | 1:10 | .2 | 1.8 | 1 drop | | 1 drop | | 100 | 4 |
| 5 | 1:100 | 1.0 | 1.0 | 1 drop | | 1 drop | | 200 | 5 |
| 6 | 1:100 | .66 | 1.34 | 1 drop | | 1 drop | | 300 | 6 |
| 7 | 1:100 | .5 | 1.5 | 1 drop | | 1 drop | | 400 | 7 |
| 8 | 1:100 | .4 | 1.6 | 1 drop | | 1 drop | | 500 | 8 |
| 9 | 1:100 | .3 | 1.7 | 1 drop | | 1 drop | | 666 | 9 |
| 10 | 1:500 | 1.0 | 1.0 | 1 drop | | 1 drop | | 1000 | 10 |
| 11 | 1:500 | .8 | 1.2 | 1 drop | | 1 drop | | 1250 | 11 |
| 12 | 1:500 | .6 | 1.4 | 1 drop | | 1 drop | | 1666 | 12 |
| 13 | 1:500 | .4 | 1.6 | 1 drop | | 1 drop | | 2500 | 13 |
| 14 | 1:500 | .2 | 1.8 | 1 drop | | 1 drop | | 5000 | 14 |
| 15 | Strep FA control | | 1.0 | 1 drop | | 1 drop | | 4+ F | 15 |
| 16 | Rabbit FA control | | 1.0 | 1 drop | | 1 drop anti rabbit FA | | No F | 16 |

INCUBATION: CUBAATSEH & DRY

TABLE 2

ANTISTREPTOLYSIN O TITRATION

| | Tube No. | Serum Dilution | Diluted Serum ml | Standard Serum Control* | Strep-tolysin O buffer, ml | Strep-tolysin O Reagent, ml | INCUBATION | Rabbit Cells 3%, ml | INCUBATION & CENTRIFUGATION | Titer in Todd Units | Tube No. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Complete Test / Screening Test | 1 | 1:10 | 1.0 | | 0.0 | 0.5 | INCUBATION | 0.5 | INCUBATION & CENTRIFUGATION | 10 | 1 |
| | 2 | 1:10 | 0.2 | | 0.8 | 0.5 | | 0.5 | | 50 | 2 |
| | 3 | 1:100 | 1.0 | 1.0 | 0.0 | 0.5 | | 0.5 | | 100 | 3 |
| | 4 | 1:100 | 0.66 | 0.66 | 0.34 | 0.5 | | 0.5 | | 150 | 4 |
| | 5 | 1:100 | 0.5 | 0.5 | 0.5 | 0.5 | | 0.5 | | 200 | 5 |
| | 6 | 1:100 | 0.4 | 0.4 | 0.6 | 0.5 | | 0.5 | | 250 | 6 |
| | 7 | 1:100 | 0.3 | 0.3 | 0.7 | 0.5 | | 0.5 | | 333 | 7 |
| | 8 | 1:500 | 1.0 | | 0.0 | 0.5 | | 0.5 | | 500 | 8 |
| | 9 | 1:500 | 0.8 | | 0.2 | 0.5 | | 0.5 | | 625 | 9 |
| | 10 | 1:500 | 0.6 | | 0.4 | 0.5 | | 0.5 | | 833 | 10 |
| | 11 | 1:500 | 0.4 | | 0.6 | 0.5 | | 0.5 | | 1250 | 11 |
| | 12 | 1:500 | 0.2 | | 0.8 | 0.5 | | 0.5 | | 2500 | 12 |
| | 13 | Streptolysin control | | | 1.0 | 0.5 | | 0.5 | | Hemolysis | 13 |
| | 14 | Red Cell Control | | | 1.5 | 0.0 | | 0.5 | | No Hemolysis | 14 |

C. Comparison of Fluorescent Antibody Blocking with Streptolysin O Antibody in Sera of Rabbits after Immunization.

Nineteen white New Zealand rabbits were immunized, nine of which died from the immunization before any meaningful data could be collected. Then ten surviving rabbits were divided into six different antigenic groups as follows:

I. Todd-Hewitt broth given to rabbits number 1 and 2 only as controls,

II. Killed and washed beta hemolytic Streptococcus group A cells given to rabbits number 3 and 4,

III. Commercial streptolysin O reagent (BioQuest cat. # 40666-68) given to rabbits numbers 5 and 6.

IV. Bacteria free filtrate of live beta hemolytic Streptococcus group A culture in Todd-Hewitt broth given to rabbit number 7,

V. Live beta hemolytic Streptococcus group A culture in Todd-Hewitt broth given to rabbits number 8 and 9, and

VI. Killed beta hemolytic Streptococcus group A culture in Todd-Hewitt broth followed by antigen V given to rabbit number 10.

Each rabbit was injected as per inoculating and bleeding schedule given in table 3.

The Todd-Hewitt Broth used was that of Updyke and Nickle's (1954) modification of Todd and Hewitt's (1932) original broth for the production of hemolysin by streptococcus. This broth is generally used for cultivation of beta hemolytic streptococci, and especially

for serological typing. Todd-Hewitt Broth (THB) was used throughout this study for the growth of streptococcal organisms.

Two strains of group A streptococci were used through-out: National Institute of Allergy and Infectious Diseases #61X101, as previously mentioned under "Organisms used", and Loma Linda University #2793 type 17 (from Merck Sharp and Dome).

Antigen II (killed streptococci) was prepared in the following manner:

1. Lyophilized cultures were reconstituted and transferred to a blood agar plate and incubated at 37° C. for 24 hours.
2. One colony was transferred to 10 ml of Todd-Hewitt broth and incubated for 16 hours,
3. One ml of the 16 hour culture was transferred to each of 4 tubes of THB and incubated at 37° C. for 4 hours.
4. At the end of 4 hours incubation, 1 ml of the above culture was diluted and plated for colony counts. (Each colony was considered to have originated from one bacterium).
5. The remainder of the 4 hour culture was centrifuged at 840 G for 5 minutes, broth decanted, and the remaining cells washed twice with 0.4% formalized normal saline and reconstituted to original volume with 0.4% formalized saline, followed by placing 0.1 ml of this cell suspension in 9 mls of THB for viability testing.
6. If there was no growth in this THB at the end of four days, the formalized cells were inoculated into the

rabbit. The volume to inoculate was determined by the estimated number of bacteria per ml as indicated in table 3.

Antigen III (streptolysin O reagent) was obtained from BBL, Division of BioQuest. Ten ml vials were used which all contained the same lot number 6031421. The reagent for each vial was rehydrated with 10 mls of sterile distilled water.

Antigen IV (filtrate of THB growth of group A streptococci) was prepared in the following way:

1 through 4. Same as for antigen II.

5. The remainder of the 4 hour culture was passed through a Millipore filter with pore size of 0.45 micron. The sediment was discarded and the filtrate was refrigerated. One ml of the filtrate was placed in 9 ml of THB and incubated over night at 37° C. to test for viability.

6. If there were no viable cells found in the filtrate, it was then inoculated into a rabbit according to the schedule in table 3. (The THB culture of filtrate in step 5 was checked for a total of seven days. No growth ever grew out in this time and the filtrates were considered sterile.) The volume of filtrate given was determined by the number of bacteria per ml in the 4 hour broth culture before filtration.

Antigen V (live group A streptococcal culture) was prepared according to the following procedure:

1 through 4. Same as for antigen II.

5. The remainder of the 4 hour culture was placed in the refrigerator and used for rabbit inoculation the next day as per schedule in table 3.

Antigen VI (heat killed group A streptococci followed by antigen V) was prepared in the following way:

1 through 4. Same as antigen II.

5. The remainder of the 4 hour culture was placed in a 56° C. water bath for two hours, followed by placing 0.1 ml of it into 9 mls of THB for viability testing.
6. If there was no growth in this viability control tube at the end of twenty-four hours, the killed streptococcal culture was inoculated into the rabbit according to the schedule given in table 3.
7. The live streptococci were prepared in the same manner as those of antigen V and given to rabbit number ten according to the schedule in table 3.

By giving killed streptococcal culture followed by live streptococcal culture, higher titers were obtained than could be obtained with just the live culture.

BLEEDING

All bleeding was done from the external marginal vein using a 23-gauge, 1-inch needle and a 10 ml vacutainer tube. The blood was allowed to clot at room temperature for two hours and further retraction was accomplished by refrigerating the blood over night, Campbell and associates (1964). After separating the clot from the

serum by centrifugation, the serum was placed in the deep freeze to be tested for FAB and ASO titers at a later date. There were a total of 157 rabbit sera samples collected for testing.

FAB and ASO titers were determined on these sera in the same manner used on the human sera outlined in tabes 1 and 2.

TABLE 3

Bleeding* and inoculating schedule for rabbits:

| TIME | INOCULATION | | | | | |
|---|---|---|---|---|---|---|
| Bleeding Days | I Todd-Hewitt Broth | | II Killed and washed group A streptococci 61x101 2793 | | III Streptolysin O BBL | |
| | Rabbit 1 | Rabbit 2 | Rabbit 3 | Rabbit 4 | Rabbit 5 | Rabbit 6 |
| 0 | 10 ml IP | 10 ml IP | $2x10^9$ IP | $2x10^9$ IP | 10 ml IP | 20 ml IP |
| 3 | | | | | | |
| 7 | | | | | | |
| 10 | | | $2x10^9$ IP | | | |
| 14 | 10 ml IP | 10 ml IP | | $2x10^9$ IP | | 30 ml IP |
| 17 | | | | | 20 ml IP | |
| 21 | | | | | | |
| 24 | | | $2x10^9$ IP | | | |
| 28 | 10 ml IP | 10 ml IP | | | | 40 ml IP |
| 31 | | | | $2x10^9$ IP | 30 ml IP | |
| 35 | | | | | | |
| 38 | | | | | | |
| 42 | | | | | | |
| 49 | | | | | | |
| 56 | | | | | | |
| 63 | | | | | | |
| 70 | | | | | | |
| 84 | | | | | | |

* Each rabbit was bled on each day enumerated in "bleeding day" column.

IP Intraperitoneal

TABLE 3 (continued)

Bleeding and inoculating schedule for rabbits:

| TIME | INOCULATION | | | TIME | INOCULATE |
|---|---|---|---|---|---|
| Bleeding Days | IV Filtrate of Strep. #61X101 | V Live Strep. #2793 | V Live Strep. #61X101 | Bleeding Days | VI Killed & Live Strep. #61X101 |
| | Rabbit 7 | Rabbit 8 | Rabbit 9 | | Rabbit 10 |
| 0 | $4x10^8$ IP | $3x10^5$ IP | $2x10^9$ IP | 0 | $2x10^8$ KIP |
| 3 | | | | 5 | |
| 7 | $2x10^8$ IP | | | 8 | $3x10^8$ KIP |
| 10 | | | | 11 | |
| 14 | $3x10^8$ IP | $4x10^7$ IP | $2x10^9$ IP | 15 | $1x10^8$ LIP |
| 17 | | | | 19 | |
| 21 | $4x10^8$ IP | | | 22 | $2x10^8$ LIP |
| 24 | | | | 25 | |
| 28 | $4x10^8$ IP | $5x10^8$ IP | $3x10^9$ IP | 29 | $3x10^8$ LIP |
| 31 | | | | 34 | |
| 35 | | | | 41 | |
| 38 | | | | 45 | $3x10^8$ LIP |
| 42 | $4x10^8$ IP | | | 48 | |
| 49 | $1x10^7$ IV | | | 52 | $1x10^7$ KIV |
| 56 | $1x10^8$ IV | | | 55 | |
| | | | | 59 | $1x10^7$ KIV |
| 63 | ** | | | 62 | |
| 70 | | | | 66 | $1x10^7$ LIV |
| 84 | | | | 90 | |
| | | | | 94 | $2x10^7$ LIV |
| | | | | 99 | |
| | | | | 100 | $3x10^7$ LIV |
| | | | | 103 | |
| | | | | 107 | $4x10^7$ LIV |
| | | | | 111 | |
| | | | | 117 | |
| | | | | 124 | |
| | | | | 130 | |
| | | | | 138 | |
| | | | | 145 | |
| | | | | 151 | |

** Rabbit died.
IP Intraperitoneal
IV Intravenous.

KIP Killed intraperitoneal.
LIP Live intraperitoneal.
KIV Killed intravenous.
LIV Live intravenous.

## RESULTS AND DISCUSSION

### A. Streptococcal immunofluorescent blocking antibody compared with the streptolysin O antibody in human patient sera.

Results of testing 100 patients sera for the presence of blocking antibodies and streptolysin O antibodies are given in table 4 and figure 7. The patients were grouped according to their ASO titer results. Only the results of testing undiluted sera for FAB are recorded in this table and graph. The titer of the streptococcal immunofluorescent blocking was not measured at this time. Blocking was considered to be present when group A streptococcal cells pretreated with the patient's serum gave only a 2+ or less fluorescence when stained with their homologous fluorescent antiserum and a 3 or 4+ fluorescence when stained with human globulin fluorescent antiserum. Thirty-four of the 100 sera had ASO titers of 50 or below with 29% of these showing some blocking. Thirty-four sera had ASO titers of 100 to 250 with 59% of these showing blocking. Thirty-two sera had ASO titers of 333 and above with 72% of these having the blocking effect. It is evident here that the per-centage of patients showing FAB increased in the groups with elevated ASO titers as illustrated in figure 7.

From the above 100 sera, sixteen sera were diluted to determine the FAB titers. The ASO titers were also re-run on these 16 sera. The results of each patient's ASO and FAB titers are compared in table 5 and the scatter diagram in figure 8. From the appearance of this scatter diagram, it was difficult to determine if there was any correlation between the ASO and the FAB titers. A statistical anal-

ysis of the data given in table 5 showed for each 1 unit change of ASO titer there was a corresponding .38 unit change (regression coefficient) of the FAB titer, table 6 and figure 8. The "y intercept" was calculated to be 80.0 FAB units. Having determined the regression coefficient and the y intercept, a straight line was made for figure 8 scatter diagram to satisfy all points. Another statistical analysis was made of the data in table 5 to determine what is the correlation coefficient (r) independent of the regression coefficient. This analysis resulted in a 0.03 r. In the other words, the linear correlationship of ASO to FAB from table 5 is small, Steel & Torrie (1960). Any r value over .5 is good correlation. The calculations for "r" and the results are given in table 6 and 7.

The first experiments on undiluted sera of the 100 patients showed promise of correlation between the ASO and the FAB. Upon diluting the sera of 16 of these patients and re-running the ASO and FAB test, the titers of the two tests did not seem to correlate. It was felt that the poor correlation was due to uncontrolled discrepancies of the sera collected from different hospitals as mentioned in the "Introduction" of this paper. In that there was difinite blocking of fluorescent antibody staining in many of the sera with elevated ASO titers, it was decided to repeat these experiments using animals in a controlled experiment.

TABLE 4

Human sera tested for ASO titer and presence of FAB.

| ASO TITER | Number of Patients | Number with FAB | % with FAB | Average % with FAB |
|---|---|---|---|---|
| under 10 | 16 | 2 | 13 | |
| 10 | 10 | 4 | 40 | 29 |
| 50 | 8 | 4 | 50 | |
| 100 | 5 | 3 | 60 | |
| 150 | 29 | 17 | 59 | 59 |
| 200 | 0 | 0 | 0 | |
| 250 | 3 | 2 | 67 | |
| 333 | 12 | 8 | 67 | |
| 500 | 0 | 0 | 0 | |
| 625 | 14 | 11 | 79 | 72 |
| 833 | 3 | 2 | 67 | |
| 1250 | 0 | 0 | 0 | |
| 2500 | 0 | 0 | 0 | |
| TOTAL | 100 | 53 | | |

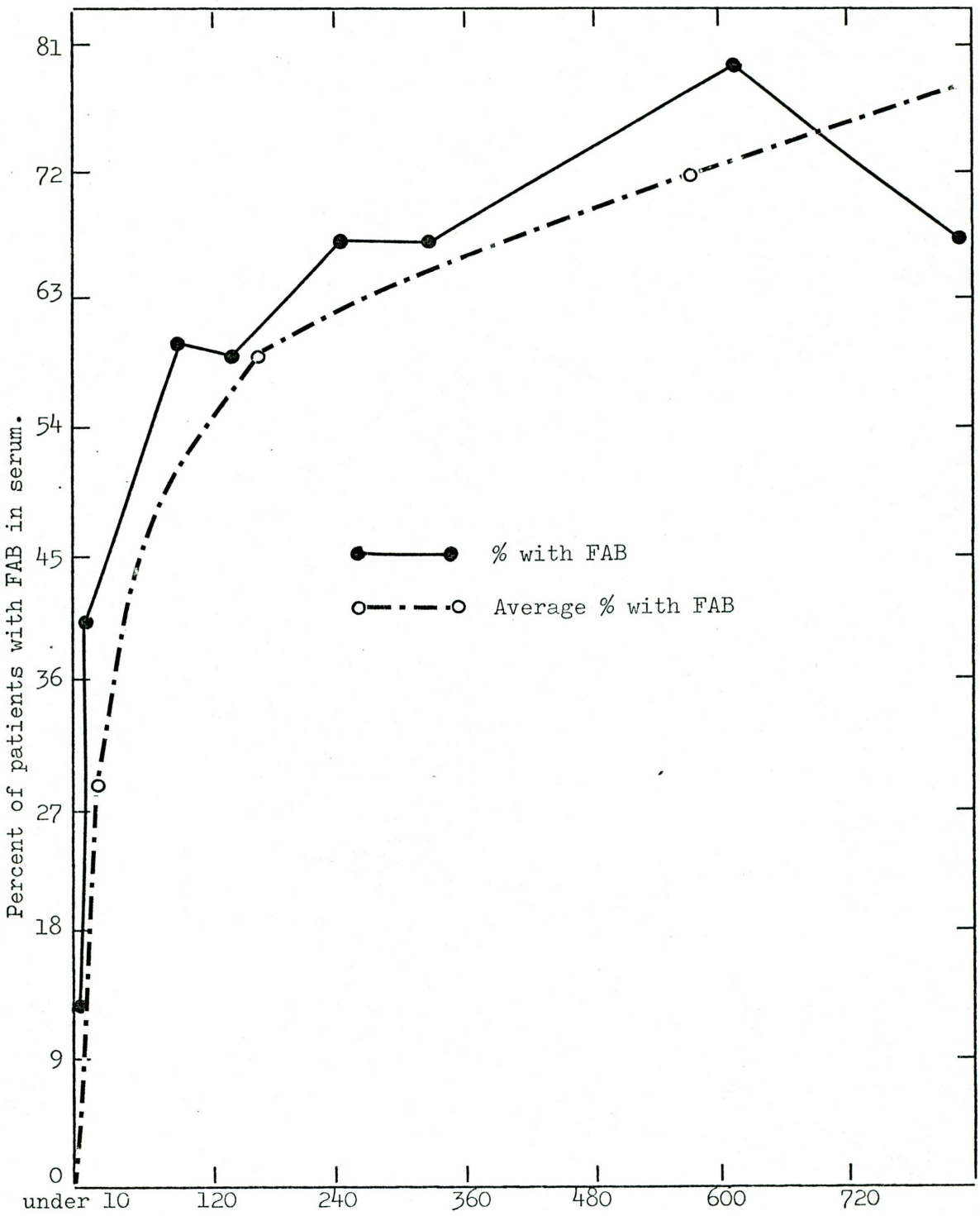


Fig. 7. Antistreptolysin O titers and fluorescent antibody blocking effect on 100 patients.

TABLE 5

FAB and ASO titers of 16 patients.

| ASO | FAB |
|---|---|
| 10 | 0 |
| 50 | 100 |
| 50 | 0 |
| 50 | 0 |
| 100 | 100 |
| 100 | 200 |
| 150 | 20 |
| 150 | 0 |
| 200 | 300 |
| 200 | 300 |
| 200 | 300 |
| 200 | 20 |
| 250 | 200 |
| 250 | 300 |
| 250 | 10 |
| 333 | 0 |

TABLE 6

Regression Coefficient from Data of Table 5.

X = ASO titer. Y = FAB titer.

| Patient Number | X | Y | $Xi - \overline{X}$ <br> Xi - 140 | $Yi - \overline{Y}$ <br> Yi - 134 | $(Xi-\overline{X}) \times (Yi-\overline{Y})$ | $(Xi-\overline{X})^2$ |
|---|---|---|---|---|---|---|
| 1 | 10 | 0 | - 130 | - 134 | 17420 | 16900 |
| 2 | 50 | 100 | - 90 | - 34 | 3060 | 8100 |
| 3 | 50 | 0 | - 90 | - 134 | 12060 | 8100 |
| 4 | 50 | 0 | - 90 | - 134 | 12060 | 8100 |
| 5 | 100 | 100 | - 40 | - 34 | 1360 | 1600 |
| 6 | 100 | 200 | - 40 | 66 | - 2640 | 1600 |
| 7 | 150 | 20 | 10 | - 114 | - 1140 | 100 |
| 8 | 150 | 0 | 10 | - 134 | - 1340 | 100 |
| 9 | 200 | 300 | 60 | 166 | 9960 | 3600 |
| 10 | 200 | 300 | 60 | 166 | 9960 | 3600 |
| 11 | 200 | 300 | 60 | 166 | 9960 | 3600 |
| 12 | 200 | 20 | 60 | - 114 | - 6840 | 3600 |
| 13 | 250 | 200 | 110 | 66 | 7260 | 12100 |
| 14 | 250 | 300 | 110 | 166 | 18260 | 12100 |
| 15 | 250 | 10 | 110 | - 124 | -13640 | 12100 |
| 16 | 333 | 0 | 193 | - 134 | -25862 | 37249 |
| SUM (Z) | 2243 | 2150 | | | 49898 | 132549 |
| average ($\overline{\ }$) | 140 | 134 | | | | |

Slope (b) = $Z(Xi - \overline{X})(Yi - \overline{Y}) / Z(Xi - \overline{X})^2$ = 49898 / 132549

= 0.38 FAB units per 1.00 ASO unit.

TABLE 6 (continued)

Regression Equation

$$Y - \overline{Y} = b(X - \overline{X}) \text{ or } y = bx$$

$$Y - 134 = .38(X - 140) \text{ or}$$

$$Y = 134 + .38(X - 140)$$

$$= 134 + .38X - 53.20$$

$$= 134 - 53.20 + .38X$$

$$= 80.80 + .38X$$

Y intercept "a" is

$$\overline{Y} - b\overline{X} = 80.8 \text{ FAB Units.}$$

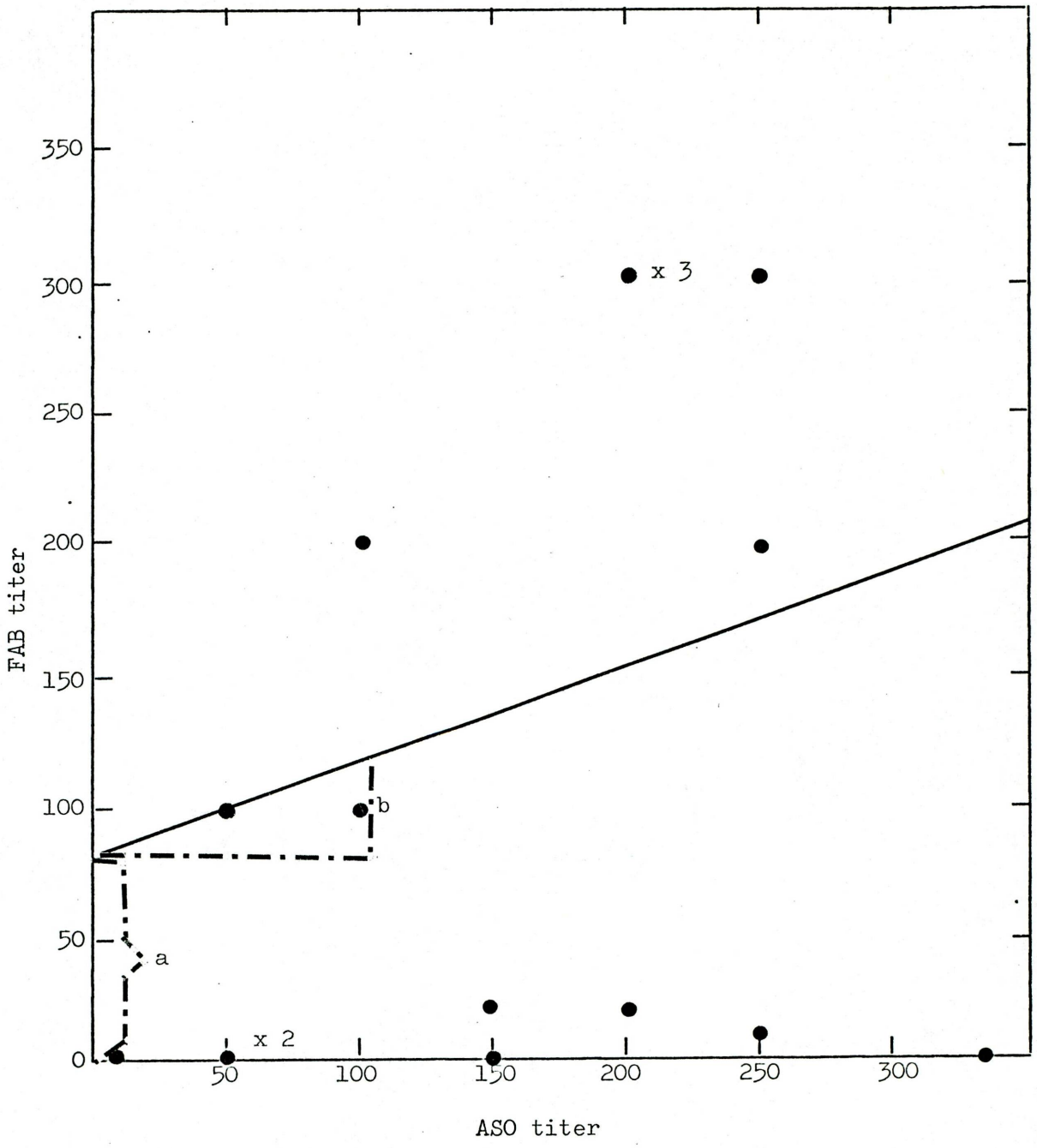


Fig. 8. Sixteen human sera titers compared.

TABLE 7

Correlation Coefficient from Data of Table 5.

| Patient Number | ASO X | FAB Y | XY | $X^2$ | $Y^2$ |
|---|---|---|---|---|---|
| 1 | 10 | 0 | 0 | 100 | 0 |
| 2 | 50 | 100 | 5000 | 2500 | 10000 |
| 3 | 50 | 0 | 0 | 2500 | 0 |
| 4 | 50 | 0 | 0 | 2500 | 0 |
| 5 | 100 | 100 | 10000 | 10000 | 10000 |
| 6 | 100 | 200 | 20000 | 10000 | 40000 |
| 7 | 150 | 20 | 3000 | 22500 | 400 |
| 8 | 150 | 0 | 0 | 22500 | 0 |
| 9 | 200 | 300 | 60000 | 40000 | 90000 |
| 10 | 200 | 300 | 60000 | 40000 | 90000 |
| 11 | 200 | 300 | 60000 | 40000 | 90000 |
| 12 | 200 | 20 | 4000 | 40000 | 400 |
| 13 | 250 | 200 | 50000 | 62500 | 40000 |
| 14 | 250 | 300 | 75000 | 62500 | 90000 |
| 15 | 250 | 10 | 2500 | 62500 | 100 |
| 16 | 333 | 0 | 0 | 110889 | 0 |
| Z | 2543 | 1850 | 349500 | 530989 | 460900 |
| $(Z)^2$ | 6466849 | 3422500 | | | |

TABLE 7 (continued)

$$r = (ZXY - (ZX)(ZY) / n) / ((ZX^2 - (ZX^2) / n)(ZY^2 - (ZY)^2 / n))^{1/2}$$

$$= (349500 - (2543)(1850) / 16) / ((530989 - 6466849 / 16)$$
$$(460900 - 3422500 / 16))^{1/2}$$

$$= (349500 - 4704550 / 16) / ((530989 - 6466849 / 16$$
$$(460900 - 3422500 / 16))^{1/2}$$

$$= (349500 - 294034.4) / ((53098 - 404178.1)(460900 - 213906.3))^{1/2}$$

$$= 55466 / ((126811)(213906))^{1/2}$$

$$= 55466 / (27403497660)^{1/2}$$

$$= 55466 / 166540.02$$

$$= 0.03.$$

In that the correlation coefficient is near zero, it is concluded that the correlation is negative, therefore an increase in ASO titer does not mean, necessarily, an increase in FAB titer.

B. Streptococcal immunofluorescent blocking antibody compared with the streptolysin O antibody in rabbit sera.

The comparison of FAB with ASO titers in human sera was unsatisfactory, as mentioned previously, therefore it was decided to study this comparison using inoculated rabbits where the factors involved could be controlled. An attempt was made to produce ASO titers as high as possible in rabbits by the use of certain antigens. ASO and FAB titers were determined on each serum sample collected and the results compared.

I. Todd-Hewitt Broth Control.

Beta hemolytic Streptococcus group A cultured in Todd-Hewitt broth was the bacterial antigen used. As a control, Todd-Hewitt broth alone was inoculated intraperitonealy into two different rabbits according to the schedule in table 3. The resulting ASO and FAB titers caused by these inoculations are given in tables 8 and 9. Neither rabbit showed any antibody reaction to the Todd-Hewitt broth inoculations. This is to say that the undiluted rabbit's sera did not prevent any fluorescent antibody staining of the streptococci and that the antistreptolysin O titers were less than 10, (tables 1 and 2).

II. Streptococcal cells suspended in formalized saline.

Two rabbits were inoculated with killed beta hemolytic Streptococcus group A cells only. These were prepared by washing and preserving the cells in 0.4% formalized saline as outlined in part "C" of Materials and Methods. The resulting ASO and FAB titers are given in tables 10 and 11. Of the 35 sera collected from these two rabbits, one collected in the fifth week showed antibody blocking and this dis-

TABLE 8

I. Todd-Hewitt Broth Antigen - Rabbit #1's response. On the day of the first inoculation, this rabbit was five months old and weighed ten pounds.

| TIME DAYS | DOSAGE | RESULTS | |
|---|---|---|---|
| | | ASO TITER | FAB TITER |
| 0 | 10 mls. | under 10 | 0 |
| 3 | | under 10 | 0 |
| 7 | | under 10 | 0 |
| 10 | | under 10 | 0 |
| 15 | 10 mls. | under 10 | 0 |
| 17 | | under 10 | 0 |
| 21 | | under 10 | 0 |
| 24 | | under 10 | 0 |
| 28 | 10 mls. | under 10 | 0 |
| 31 | | under 10 | 0 |
| 36 | | under 10 | 0 |
| 38 | | under 10 | 0 |
| 49 | | under 10 | 0 |
| 57 | | under 10 | 0 |
| 63 | | under 10 | 0 |
| 70 | | under 10 | 0 |
| 84 | | under 10 | 0 |

TABLE 9

I. Todd-Hewitt Broth Antigen - Rabbit #2's response. On the day of the first inoculation, this rabbit was five months old and weighed six pounds.

| TIME DAYS | DOSAGE | RESULTS | |
|---|---|---|---|
| | | ASO TITER | FAB TITER |
| 0 | 10 mls. | under 10 | 0 |
| 3 | | under 10 | 0 |
| 7 | | under 10 | 0 |
| 10 | | under 10 | 0 |
| 15 | 10 mls. | under 10 | 0 |
| 17 | | under 10 | 0 |
| 21 | | under 10 | 0 |
| 24 | | under 10 | 0 |
| 28 | 10 mls. | under 10 | 0 |
| 31 | | under 10 | 0 |
| 36 | | under 10 | 0 |
| 38 | | under 10 | 0 |
| 49 | | under 10 | 0 |
| 57 | | under 10 | 0 |
| 63 | | under 10 | 0 |
| 70 | | under 10 | 0 |
| 84 | | under 10 | 0 |

appeared when diluted beyond 1:10. The response of the killed streptococcal cells was practically the same as for the Todd-Hewitt broth antigen, which is the negative control.

III. Streptolysin O reagent, Commercially Prepared.

Two rabbits were chosen to determine the response when commercially prepared streptolysin O reagent was used as the antigen. This extract was obtained from BBL Division of BioQuest. The first rabbit received injections of 10, 20 and 30 mls of the reagent as shown in tables 3 and 12. The FAB rose only slightly after the third injection and rapidly returned to zero. The ASO titers in the same rabbit rose to a titer of 10 and remained at this level through the remaining 12 weeks. It was thought that higher inoculation doses would give more marked responses. The second rabbit received injections of 20, 30 and 40 mls of the reagent as shown in tables 3 and 13. Surprisingly, this second rabbit showed no FAB response, (table 13). The ASO response increased to a titer of 50 and then leveled off to the same level as the previous one during the last five weeks of the study. In comparing the results of the last two rabbits mentioned, it is noted that the ASO levels rose at practically the same time. There was a definate ASO response to the streptolysin O reagent but of a low titer. Apparently, the commercially prepared streptolysin O reagent used in the ASO titration, table 2, is of low titer. This researcher was unable to obtain from the BioQuest Company their method of determining the concentration of streptolysin O in this reagent.

IV. Cell free filtrate of streptococcal broth culture.

It was thought that a specially prepared streptococcal broth

TABLE 10

II. Streptococcal cells suspended in formalized saline - Rabbit #3's response. On the day of the first inoculation, this rabbit was three months old and weighed seven pounds.

| TIME DAYS | DOSAGE: TOTAL NUMBER OF STRAIN 61X101 STREPTOCOCCAL CELLS | RESULTS | |
|---|---|---|---|
| | | ASO TITER | FAB TITER |
| 0 | $2 \times 10^9$ | under 10 | 0 |
| 3 | | under 10 | 0 |
| 6 | | under 10 | 0 |
| 10 | $2 \times 10^9$ | under 10 | 0 |
| 13 | | under 10 | 0 |
| 17 | | under 10 | 0 |
| 20 | | under 10 | 0 |
| 24 | $2 \times 10^9$ | under 10 | 0 |
| 27 | | under 10 | 0 |
| 31 | | under 10 | 10 |
| 34 | | under 10 | 0 |
| 39 | | under 10 | 0 |
| 46 | | under 10 | 0 |
| 52 | | under 10 | 0 |
| 59 | | under 10 | 0 |
| 66 | | under 10 | 0 |
| 80 | | under 10 | 0 |

TABLE 11

II. Streptococcal cells suspended in formalized saline - Rabbit #4's response. On the day of the first inoculation, this rabbit was four months old and weighed eight pounds.

| TIME DAYS | DOSAGE: TOTAL NUMBER OF STRAIN 2793 STREPTOCOCCAL CELLS | RESULTS | |
|---|---|---|---|
| | | ASO TITER | FAB TITER |
| 0 | $2 \times 10^9$ | under 10 | 0 |
| 3 | | under 10 | 0 |
| 7 | | under 10 | 0 |
| 10 | | under 10 | 0 |
| 15 | $2 \times 10^9$ | under 10 | 0 |
| 17 | | under 10 | 0 |
| 22 | | under 10 | 0 |
| 24 | | under 10 | 0 |
| 28 | | under 10 | 0 |
| 32 | $2 \times 10^9$ | under 10 | 0 |
| 35 | | under 10 | 0 |
| 39 | | under 10 | 0 |
| 42 | | under 10 | 0 |
| 49 | | under 10 | 0 |
| 56 | | under 10 | 0 |
| 63 | | under 10 | 0 |
| 71 | | under 10 | 0 |
| 85 | | under 10 | 0 |

culture filtrate might contain a higher concentration of streptolysin O than the commercially prepared reagent mentioned above. The filtrate was prepared by passing a four hour Todd-Hewitt broth culture through a Millipore filter with a pore size of .45 microns. (See part C of Materials and Methods.) Only one rabbit survived inoculations with this antigen long enough to collect any meaningful data. This number seven rabbit's inoculation and bleeding schedules are given in table 3 and 14. An attempt was made to obtain higher ASO and FAB titers by giving more frequent inoculations of the antigen. The first six inoculations were given intraperitoneally and the last two intravenous. The results of inoculating this filtrate are given in table 14 and figure 9. The ASO and FAB titers rose sooner and went much higher than with the streptococcal cells or the streptolysin O reagent. Both titers began to rise at the same time after the third inoculation or during the third week. The FAB titer rose to 2500 in the sixth week whereas the ASO titer rose to a peak of 1250 in the eighth week. The FAB titer began to drop in the eighth week and the ASO titer began to fall in the ninth week. In the ninth week, the rabbit succumbed, supposedly, due to the bacterial filtrate inoculations. In the last week, the rabbit showed signs of severe weight lose, loose stools and stiffness of its hind quarters.

Higher titers of ASO and FAB were obtained using the bacterial filtrate than with any of the other antigens. There seemed to be an immunological paralysis just before death. This is evidenced by the fact that the last two inoculations of antigen caused a falling of the FAB titer instead of rising, and the last inoculation of the antigen had a simular effect on the ASO titer. These last two inoculations were

TABLE 12

III. Commercially prepared Streptolysin O reagent - Rabbit #5's response. On the day of the first inoculation, this rabbit was four months old and weighed seven pounds.

| TIME DAYS | DOSAGE | RESULTS | |
|---|---|---|---|
| | | ASO TITER | FAB TITER |
| 0 | 10 mls | under 10 | 0 |
| 4 | | under 10 | 0 |
| 10 | | under 10 | 0 |
| 14 | | under 10 | 0 |
| 17 | 20 mls | under 10 | 0 |
| 21 | | under 10 | 0 |
| 24 | | under 10 | 0 |
| 28 | | under 10 | 0 |
| 31 | | under 10 | 0 |
| 32 | 30 mls | under 10 | 0 |
| 35 | | under 10 | 0 |
| 38 | | 10 | 10 |
| 43 | | 10 | 10 |
| 45 | | 10 | 0 |
| 52 | | 10 | 0 |
| 59 | | 10 | 0 |
| 66 | | 10 | 0 |
| 73 | | 10 | 0 |
| 87 | | 10 | 0 |

TABLE 13

III. Commercially prepared Streptolysin O reagent - Rabbit #6's response. On the day of the first inoculation, this rabbit was four months old and weighed eight pounds.

| TIME DAYS | DOSAGE | RESULTS | |
|---|---|---|---|
| | | ASO TITER | FAB TITER |
| 0 | 20 mls | under 10 | 0 |
| 3 | | under 10 | 0 |
| 7 | | under 10 | 0 |
| 10 | | under 10 | 0 |
| 14 | 30 mls | under 10 | 0 |
| 17 | | under 10 | 0 |
| 21 | | under 10 | 0 |
| 24 | | under 10 | 0 |
| 28 | 40 mls | under 10 | 0 |
| 31 | | under 10 | 0 |
| 36 | | 50 | 0 |
| 38 | | 50 | 0 |
| 42 | | 50 | 0 |
| 49 | | 10 | 0 |
| 56 | | 10 | 0 |
| 63 | | 10 | 0 |
| 70 | | 10 | 0 |
| 84 | | 10 | 0 |

given intravenously and therefore caused a greater shock to the immunological system than the previous intraperitoneal injections had.

V. Live streptococci in Todd-Hewitt broth.

As previously mentioned in the Introduction, a major purpose in this study was to compare the ASO results with the FAB results in sera of animals infected with group A beta hemolytic streptococci. Antigens I through IV were used to determine which component of a streptococcal culture caused the highest elevation of ASO and FAB titers. Antigen V (live streptococci) were used to produce a simulated streptococcal infection as might be found in human patients who develop rheumatic fever and have elevated ASO titers. This antigen consisted of live Streptococcus pyogenes group A organisms in Todd-Hewitt broth. The antigen was prepared, standardized and checked for purity as discribed in part "C" of Materials and Methods. Nine rabbits in all received this antigen. Of these, only two survived injections long enough to collect meaningful data.

The results of number eight rabbit's response to this antigen is given in table 15 and figure 10. It might be noted here that the FAB titer rose ten days after the first inoculation whereas the ASO titer rose eleven days after the second inoculation. The FAB titer rose to a high of 200 seven days after the third and last inoculation whereas the ASO titer rose to a high of 50 at the same time. The FAB titer returned to zero 28 days after the third and last inoculation whereas the ASO titer returned to 10 fourteen days after the third inoculation and remained at 10 throughout the remainder of the 12 weeks of the experiment.

The other rabbit, number nine, that survived the live streptococcal

TABLE 14

IV. Cell free filtrate of streptococcal broth culture - Rabbit #7's response. On the day of the first inoculation, this rabbit was three months old and weighed six pounds.

| TIME DAYS | DOSAGE Total number of Bacteria* in Broth before Filtration. | RESULTS ASO TITER | RESULTS FAB TITER |
|---|---|---|---|
| 0 | $4 \times 10^8$ IP | under 10 | 0 |
| 3 | | under 10 | 0 |
| 6 | $2 \times 10^8$ IP | | |
| 11 | | under 10 | 0 |
| 14 | $3 \times 10^8$ IP | | |
| 17 | | 10 | 20 |
| 21 | $4 \times 10^8$ IP | | |
| 25 | | 50 | 100 |
| 28 | $4 \times 10^8$ IP | | |
| 31 | | 250 | 666 |
| 39 | | 625 | 2500 |
| 43 | $4 \times 10^8$ IP | | |
| 46 | | 833 | 2500 |
| 50 | $1 \times 10^7$ IV | | |
| 53 | | 1250 | 1250 |
| 57 | $1 \times 10^8$ IV | | |
| 60 | | 250 | 400 |
| 62 | | ** | ** |

* Strain 61X101 beta Streptococcus

** Rabbit died.

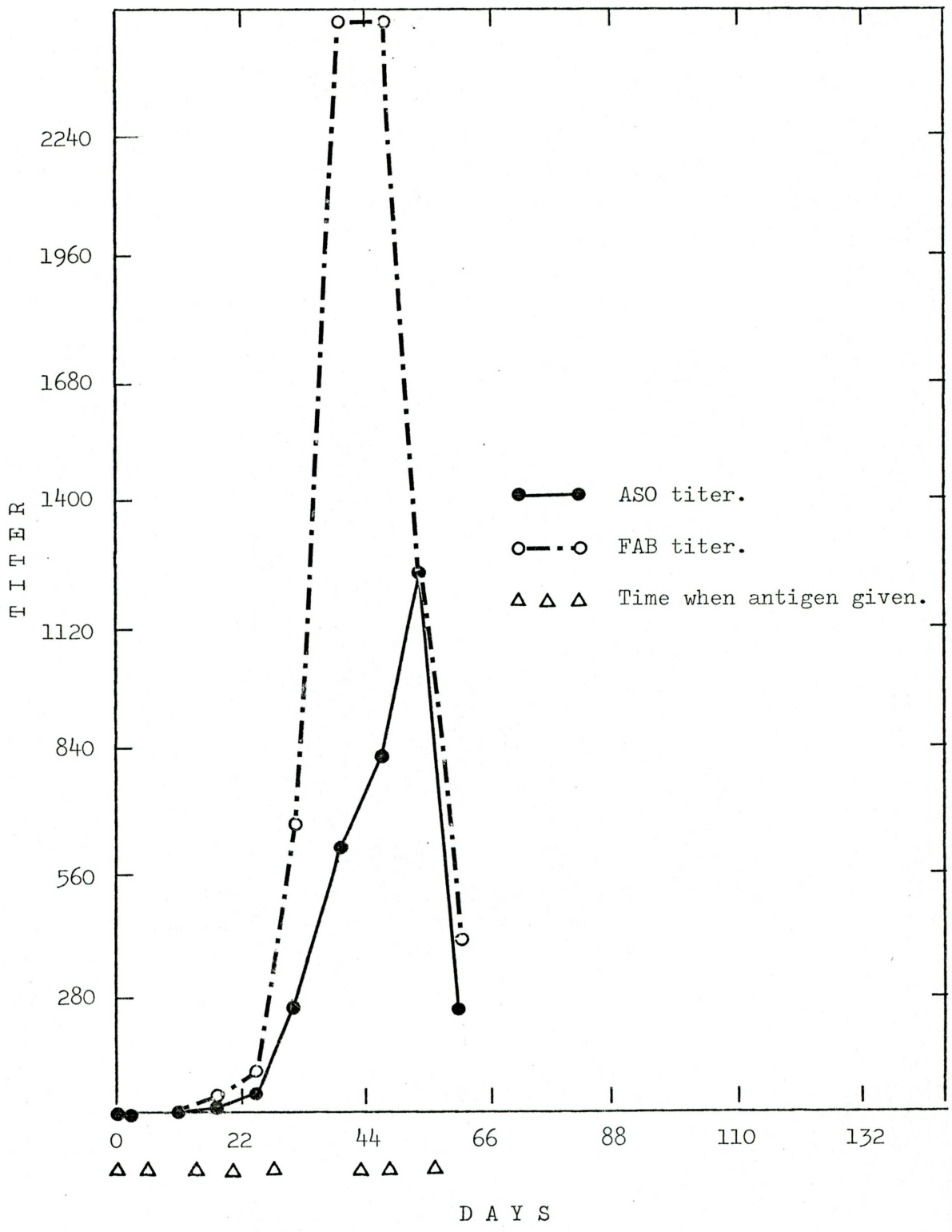


Fig. 9. IV. Cell free filtrate of streptococcal broth culture - Rabbit #7's response. (Strain 61X101 Streptococcus)

inoculations received much higher doses of this antigen than did number eight rabbit. Each inoculation that number nine received was approximately ten fold more concentrated than the highest dose number eight rabbit received. The immunological response was correspondingly greater as can be seen in table 16 and figure 11. The FAB began to rise three days after the second inoculation whereas the ASO titer began to rise seven days after the second inoculation. The FAB titer rose to a high of 500 fourteen days after the third inoculation whereas the ASO titer rose to a high of 100 twenty-eight days after this last inoculation. The FAB titer fell to zero 56 days after the last inoculation whereas the ASO titer fell to 10 forty-two days after this inoculation and remained so through-out the twelve weeks of the experiment.

By comparing the results of number eight and nine rabbit's immunization with live streptococci as observed in figure 12, it can be seen graphically that the FAB titer rises earlier, rises higher and returns to normal earlier than the ASO titer. Although definite FAB and ASO responses were observed in these two rabbits, elevated ASO titers as found in human patients with rheumatic fever were not obtained. According to Roy (1956), ASO titers below 159 are not usually indicative of rheumatic fever. The highest ASO titer obtained from either of the above two rabbits was 100. An attempt was made to produce higher ASO titers by giving larger doses of live streptococci. This attempt was unsuccessful due to the fact that the rabbits would not tolerate larger doses of this antigen than given to the previous two rabbits.

VI. Killed streptococci followed by live streptococci.

It was thought that the rabbit could be protected from succumbing

TABLE 15

V. Live streptococci in Todd-Hewitt broth - Rabbit #8's response. On the day of the first inoculation, this rabbit was three months old and weighed seven pounds.

| TIME DAYS | DOSAGE TOTAL NUMBER OF STRAIN 2793 beta STREPTO-COCCUS. | RESULTS | |
|---|---|---|---|
| | | ASO TITER | FAB TITER |
| 0 | $3 \times 10^5$ | under 10 | 0 |
| 3 | | under 10 | 0 |
| 7 | | under 10 | 0 |
| 10 | | under 10 | 10 |
| 14 | $4 \times 10^7$ | under 10 | 10 |
| 17 | | under 10 | 10 |
| 21 | | under 10 | 50 |
| 25 | | 10 | 50 |
| 28 | $5 \times 10^8$ | 10 | 50 |
| 31 | | 10 | 100 |
| 35 | | 50 | 200 |
| 38 | | 50 | 200 |
| 42 | | 10 | 20 |
| 49 | | 10 | 20 |
| 56 | | 10 | 0 |
| 63 | | 10 | 0 |
| 70 | | 10 | 0 |
| 84 | | 10 | 0 |

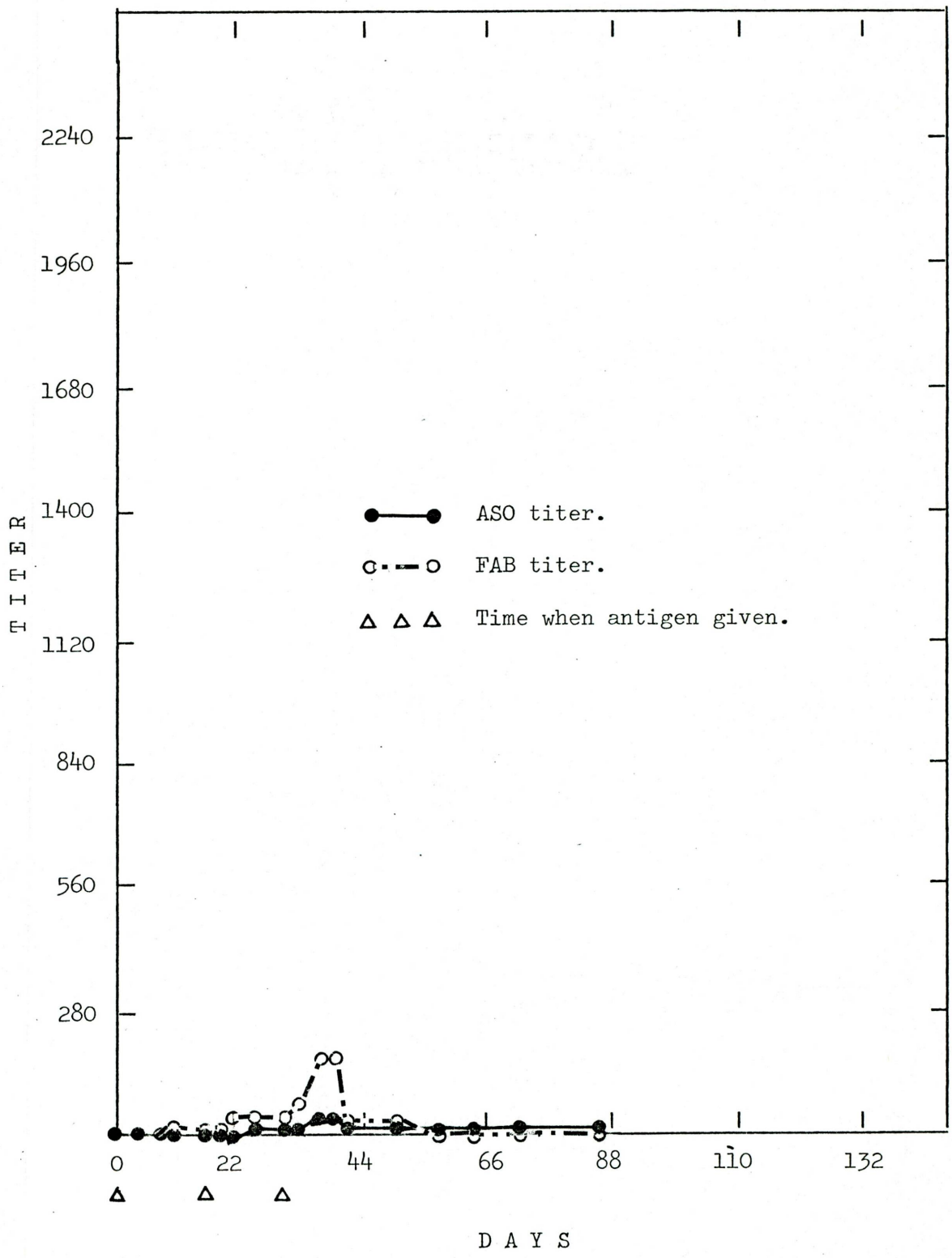


Fig. 10. V. Live Streptococci in Todd-Hewitt broth - Rabbit #8's response. (Strain 2793 beta Streptococcus.)

TABLE 16

V. Live streptococci in Todd-Hewitt broth - Rabbit #9's response. On the day of the first inoculation, this rabbit was three months old and weighed seven pounds.

| TIME DAYS | DOSAGE: TOTAL NUMBER OF STRAIN 61X101 beta STREPTOCOCCUS. | RESULTS | |
|---|---|---|---|
| | | ASO TITER | FAB TITER |
| 0 | $2 \times 10^9$ | under 10 | 0 |
| 3 | | under 10 | 0 |
| 7 | | under 10 | 0 |
| 10 | | under 10 | 0 |
| 14 | $2 \times 10^9$ | under 10 | 0 |
| 17 | | under 10 | 10 |
| 21 | | 10 | 10 |
| 24 | | 10 | 10 |
| 28 | $3 \times 10^9$ | 10 | 100 |
| 31 | | 10 | 100 |
| 35 | | 50 | 200 |
| 38 | | 50 | 400 |
| 42 | | 50 | 500 |
| 49 | | 100 | 400 |
| 56 | | * | 100 |
| 63 | | 50 | 100 |
| 70 | | 10 | 10 |
| 84 | | 10 | 0 |

* Serum quantity not sufficient to run test.

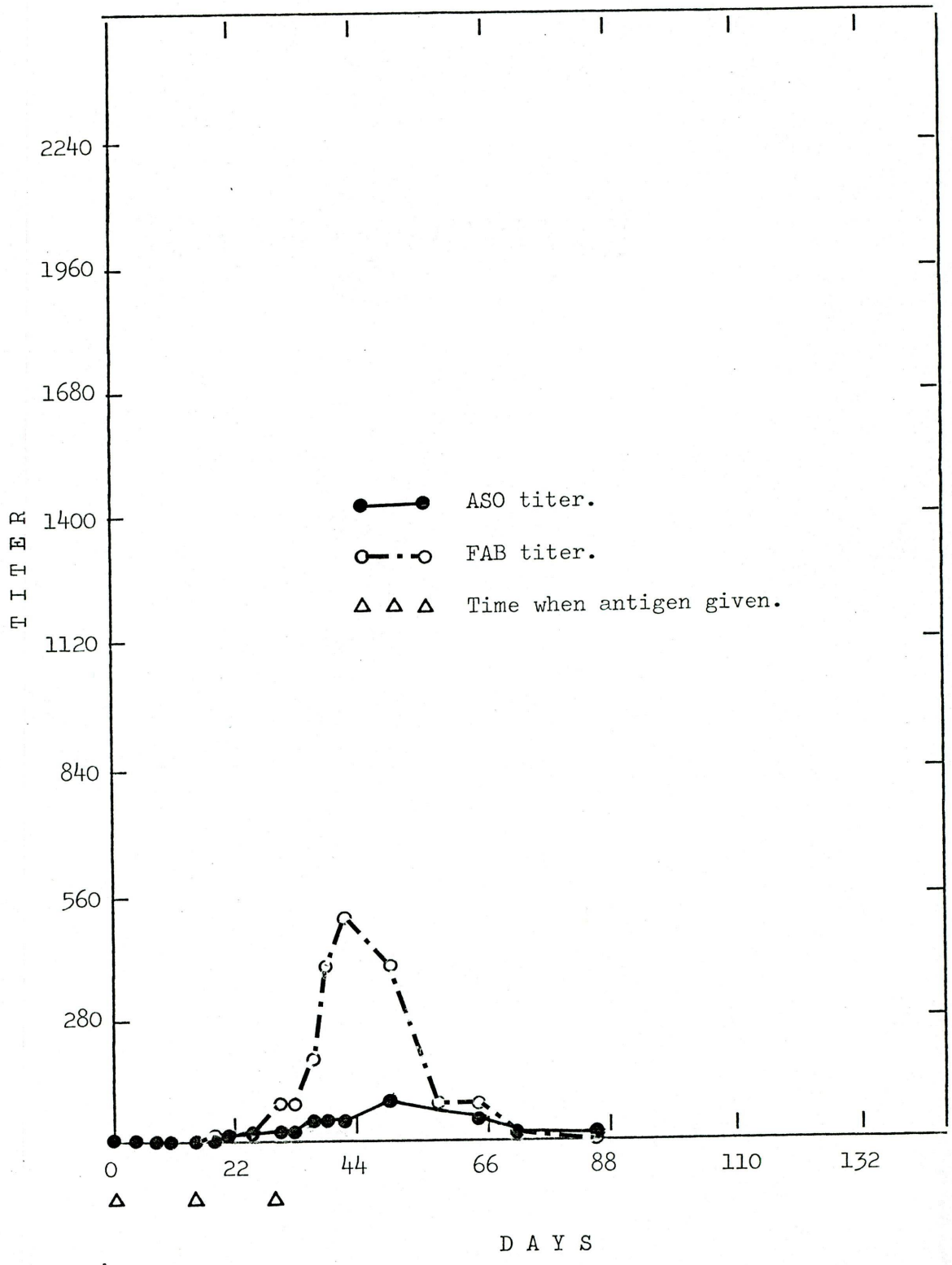


Fig. 11. V. Live Streptococci in Todd-Hewitt broth - Rabbit #9's response. (Strain 61X101 streptococci)

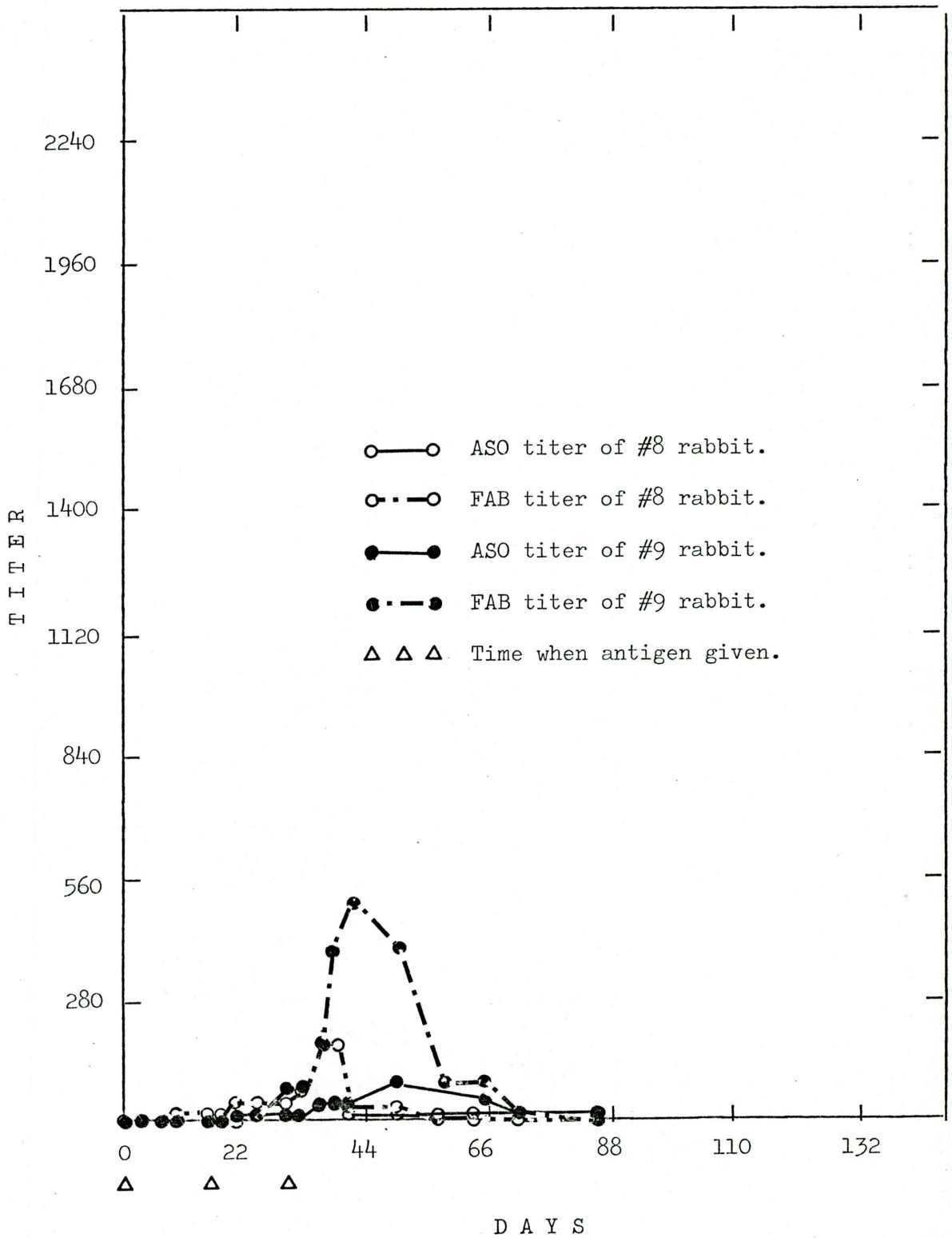


Fig. 12. V. Live streptococci in Todd-Hewitt broth. Rabbits #8 and #9's responses compared.

to higher doses of live streptococci than previously given by first giving inoculations of killed streptococci. This was accomplished by using a method similar to that used by Lancefield (1928). The killed organisms consisted of a Todd-Hewitt broth culture of streptococci which had been heated at 56° C. for 2 hours as discribed in part "C" of Materials and Methods. These were not washed cells as those for antigen II. The live streptococci were prepared in the same manner as those for antigen V. This protocol VI, killed streptococci followed by live streptococci antigen, was inoculated into only one rabbit (number 10) in the following sequence:

Two inoculations of heat killed streptococci in Todd-Hewitt broth given intraperitoneally,

Five inoculations of live streptococci in Todd-Hewitt broth given intraperitoneally,

Two inoculations of heat killed streptococci in Todd-Hewitt broth given intravenously,

Four inoculations of live streptococci in Todd-Hewitt broth given intravenously.

Each intraperitoneal inoculation was $10^8$ organisms whereas each intravenous inoculation was $10^7$ organisms.

Although the total number of organisms rabbit 10 received was no greater than other rabbits that had received streptococci, the immune response was much greater. One out-standing difference was that rabbit 10 was the only one to tolerate live streptococci given intravenously.

The ASO and FAB test results are recorded in table 17 and figure 13. Three days after the last intravenous inoculation of live streptococci, the ASO titer rose to a peak of 500 which is well into the range

of ASO titers found in patients with rheumatic fever, Roy (1956). At the same time the ASO titer reached a peak, the FAB titer reached a peak of 1666. The titer of the FAB began to rise three weeks before the ASO titer began to rise. The ASO and FAB titers began to fall at the same time but at different rates. Twenty days after the peak, the ASO titer fell to 10 and the FAB titer fell to 1250. Thirty-five days after the peak, the last time this rabbit was bled for tests, the ASO titer was still 10 and the FAB titer had dropped to 400. It was also demonstrated in this rabbit, as with the two that received live streptococci and the one that received sterile filtrate from streptococcal culture, that the FAB titer rises earlier and reaches a higher level than the ASO titer. With two rabbits that received live streptococci only, FAB titers returned to zero before the ASO titers returned to less than ten. It is assumed that the FAB titers of rabbit 7 (antigen IV) and rabbit 10 (antigen VI) would have also returned to zero before the ASO titers returned to less than ten had the FAB and ASO been tested for beyond the time limit that they were.

In comparing the immune responses to antigen IV and VI, as shown in figure 14, it is noted that the FAB and ASO titers of IV were respectively 2500 and 1250 whereas those of VI were 1666 and 500. Both antigens were from the same strain of group A beta hemolytic streptococci and given in approximately the same concentration, (tables 14 and 17). White New Zealand rabbits of the same age and body weight were used for both antigens. The only appreciable difference of the two experiments was that IV antigen consisted of broth culture filtrate only whereas the VI antigen consisted of both broth culture filtrate plus live bacterial cells. It appears that the streptococcal broth

culture cell free filtrate has a more pronounced ASO and FAB response than the complete streptococcal culture. The possible reason for this difference, along with other findings, will be discussed in the conculsion portion of this paper.

TABLE 17

VI. Response to strain 61X101 killed streptococci followed by the same strain of live streptococci - Rabbit #10's response. On the day of the first inoculation, this rabbit was three months old and weighed six pounds.

| TIME DAYS | DOSAGE TOTAL NUMBER OF BACTERIA | RESULTS ASO TITER | RESULTS FAB TITER |
|---|---|---|---|
| 0 | $2 \times 10^8$ DEAD IP | under 10 | 0 |
| 5 | | under 10 | 0 |
| 8 | $3 \times 10^8$ " " | | |
| 11 | | under 10 | 0 |
| 15 | $1 \times 10^8$ LIVE IP | | |
| 19 | | under 10 | 20 |
| 22 | $2 \times 10^8$ " " | | |
| 25 | | under 10 | 100 |
| 29 | $3 \times 10^8$ " " | | |
| 34 | | under 10 | 200 |
| 41 | | under 10 | 200 |
| 45 | $3 \times 10^8$ | | |
| 48 | | 10 | 200 |
| 52 | $1 \times 10^7$ DEAD IV | | |
| 55 | | 10 | 300 |
| 59 | $1 \times 10^7$ " " | | |
| 62 | | 10 | 500 |
| 66 | $1 \times 10^7$ LIVE IV | 10 | 500 |
| 90 | | 50 | 666 |
| 94 | $2 \times 10^7$ " " | | |
| 99 | | 150 | 666 |
| 100 | $3 \times 10^7$ " " | | |
| 103 | | 250 | 1250 |
| 107 | $4 \times 10^7$ " " | | |
| 110 | | 500 | 1666 |
| 117 | | 50 | 1250 |
| 124 | | 50 | 1250 |
| 130 | | 10 | 1250 |
| 138 | | 10 | 500 |
| 145 | | 10 | 400 |

IP = Intraperitoneal
IV = Intravenous

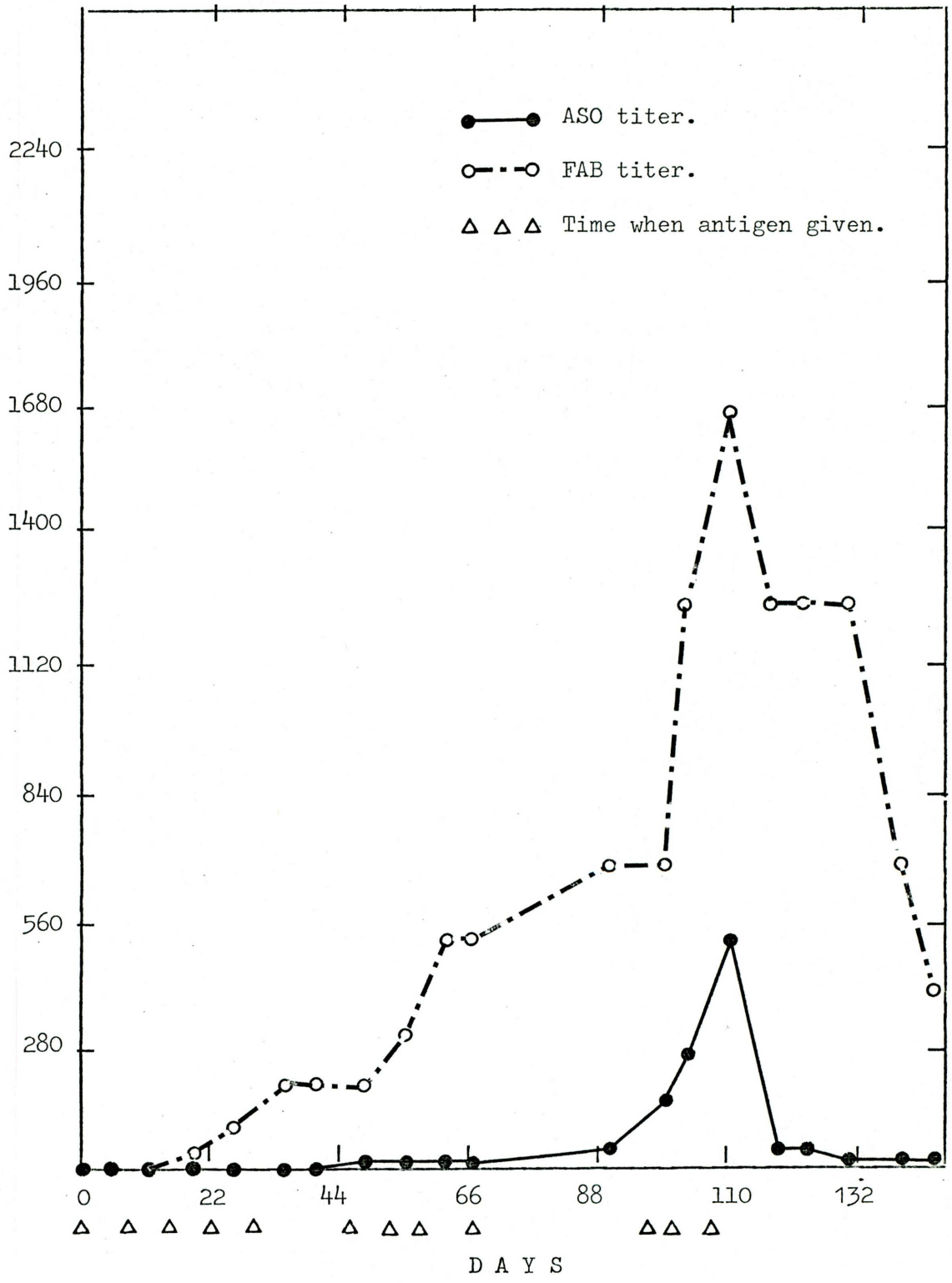


Fig. 13. VI. Strain 61X101 killed streptococci followed by the same strain of live streptococci - Rabbit #10's response.

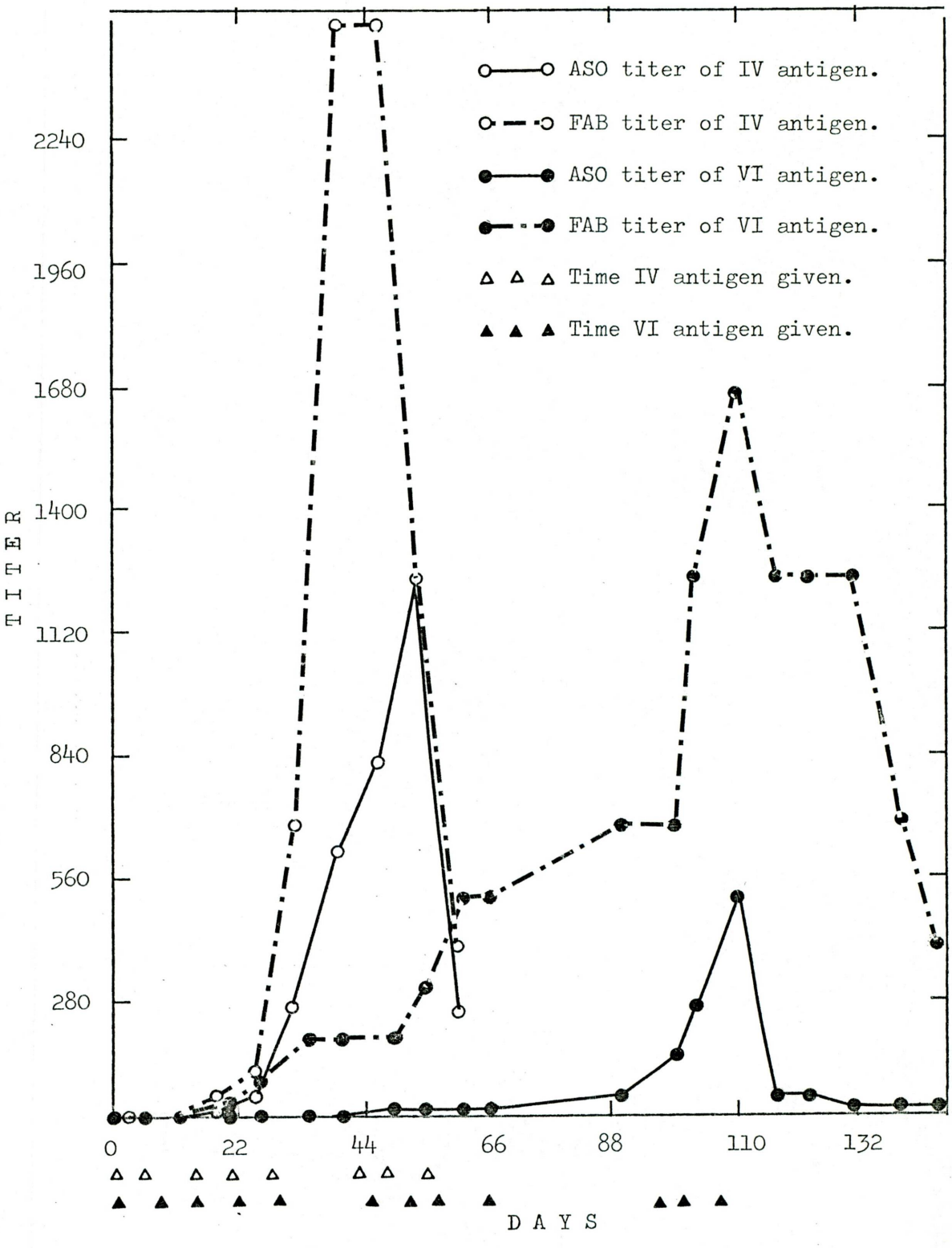


Fig. 14. IV antigen's immunological response compared to VI antigen's immunological response.

## CONCLUSIONS

The purpose of this investigation was to determine if the blocking in the group A streptococcal fluorescent antibody test might be the same as the antistreptolysin O (ASO) antibody. Two sets of experiments were performed. The first using certain human patient sera collected through the clinical laboratory and the second using sera from rabbits which had been immunized with various group A streptococcal antigens.

It was determined, using the human patient sera, that the probability of an individual having the fluorescent antibody blocking (FAB) substance is higher in those with elevated ASO titers than those with low or no ASO titer, (table 4 and figure 7). However, there was poor correlation between the two test results as illustrated in figure 8. Several uncontrollable factors possibly accounting for this discrepancy are:

a. The FAB and the ASO are possibly not the same substance.

b. It is known that a certain percent of sera produce false positive ASO titers, Packalen (1948).

c. Undetermined amount of deteriation of protein due to improper storage of sera supplied by the clinical laboratories.

It was therefore decided that a more meaningful comparison of FAB and ASO titers could be obtained under a more controlled program. Four month old white New Zealand rabbits were inoculated with various group A streptococcal antigens for this purpose. The question presented in the purpose of this study, as well as other questions in relation to the FAB and the ASO, have been partially answered by the results of these controlled animal experiments.

The FAB and the ASO are similar in some respects but differ in others. It is believed that these differences are enough to conclude that the FAB substance and the ASO are not the same.

One similarity is that both FAB and ASO titers rise at the time of a streptococcal infection. Both titers rose in the sera of rabbits inoculated with live streptococci, (figures 10, 11 and 13).

Evidence from this study supports the theory that the FAB substance is also an antibody. In the first place, this substance acts like a protein. For instance, it can be readily removed from streptococcal cells with 0.1 N sodium hydroxide. Secondly, the FAB substance acts like an antibody. As evidenced in this experiment, it took two to four weeks for the FAB substance to appear in the rabbits' sera after the initial contact with the streptococcal antigen.

Another similarity is that both the FAB and ASO antibodies are stimulated by antigens in the exudate of a streptococcal culture. Neither antibody appeared in rabbits inoculated with the washed streptococcal cell antigen. Both antibody titers appear when rabbits were inoculated with live streptococcal cultures or cell free filtrate of live streptococcal cultures, (figures 9, 10, 11 and 13).

The FAB antibody and the ASO differ in that:

a. Inoculation of streptolysin O antigen into rabbits did not stimulate the production of FAB antibodies (FABA) but did produce streptolysin O antibodies, (tables 12 and 13).

b. The FABA appeared earlier than the ASO in each of the three rabbits inoculated with live streptococci, the FABA titer being 1:50 or above in three to four weeks whereas the ASO

titer reached this titer only after seven to thirteen weeks after the initial inoculation.

c. The FABA titer goes higher in a streptococcal infection. The FABA titer rose approximately four times higher than the ASO titer in each of the three rabbits receiving live streptococci and twice as high in the rabbit receiving cell free filtrate of a streptococcal culture, (figures 9, 10, 11 and 13).

d. The ASO antibodies (ASOA) remain in the sera of immunized rabbits longer than the FABA. In the two rabbits receiving live streptococci culture only, the FABA titer returned to zero four to five weeks after the last inoculation of antigen whereas the ASOA titer was still 1:10 eight weeks after the initial inoculation. The FABA titer fell to zero at a rate proportional to the height of the titer curve, (figures 10 and 11).

From the foregoing findings, it can be concluded that the FAB substance is an antibody dissimilar from that of the ASOA. The uniqueness of this research has been to measure the titer of FABA in rabbits immunized with various streptococcal antigens and to compare these results with the ASO test results on the same sera samples. It has been beyond the scope of this project to characterize which fraction of the streptococcal antigen complex stimulates the production of FABA. This endeaver would be an interesting follow-up research project. However, it is evident that the antigen producing the FAB effect is in the streptococcal exudate as shown by the fact that the highest FABA

titer was produced from immunizing with exudate only whereas washed streptococcal cells produced no FABA titer. It has been concluded that streptolysin O is not the antigen involved here and it can not be streptolysin S as it is non-antigenic of itself, Todd (1938).

A practical aspect derived from the results of this research has been the discovery that the FABA titer rises earlier and goes higher than the ASO titer in streptococcal infections. Possibly the FABA test could be used as a diagnostic tool for the early detection of streptococcal antibodies in patient's serum and serve better than the ASO test.

SUMMARY

Human patient's sera and sera of streptococcal immunized rabbits were tested for the presence and titer of fluorescent antibody blocking (FAB) substance and anti-streptolysin O (ASO) antibody. From the results of this experiment, it is evident that both FAB and ASO are elevated in streptococcal infections but that they are not the same substance. The FAB substance has the characteristic of an antibody and has been referred to as this in the later part of this report. This FAB antibody (FABA) titer rises earlier and goes higher than the ASO titer and therefore might be a better diagnostic test for the early detection of streptococcal antibodies in sera of patients. The antigen stimulating the production of FABA has proven to be some portion of the beta hemolytic group A streptococcal exudate other than the streptolysins.

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# IMMUNE BLOCKING OF FLUORESCENT ANTIBODY STAINING OF STREPTOCOCCUS GROUP A COMPARED WITH ANTI-STREPTOLYSIN O

by

George K. Peck

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An Abstract of a Thesis

in Partial Fulfillment of the Requirements

for the Degree Master of Science

in the Field of Microbiology

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June 1970

ABSTRACT

The fluorescent antibody blocking antibody (FABA) test has been discribed and compared with the conventional antistreptolysin O antibody (ASOA) test in the detection of Streptococcus pyogenes group A antibodies in sera samples.

A slide inhibition test was used to detect the presence of FABA in the following manner; (a) fix group A streptococcal cells to a slide, (b) incubate these cells with the patient's serum for thirty minutes, (c) wash off serum with buffered saline and dry, (d) incubate these same cells with group A streptococcal fluorescent tagged antibody for thirty minutes, (e) wash and dry as before, and (f) mount and examine slide with a fluorescent microscope. The presence of streptococcal antibodies in the patients serum will block the staining of the cells with the fluorescent tagged antibody. The FABA titer can be determined by diluting the serum until there is no more blocking effect.

Sera from one hundred hospital patients were tested for the presence of FABA and ASOA titers. The probability of a patient having FABA increased in those with elevated ASOA titers, although there was poor correlation of the titers of the two tests.

The above experiment was followed by a more controlled experiment using rabbits immunized with different group A streptococcal antigens. The antigens were given as follows; (a) two rabbits received Todd-Hewitt broth (THB) as a control, (b) two rabbits received washed group A streptococcal cells, (c) two rabbits received streptolysin O reagent, (d) one rabbit received a THB cell free filtrate of a group A streptococcal culture, (e) two rabbits received live cultures of

group A streptococci in THB, and (f) one rabbit received heat killed streptococcal cells in the THB followed by antigen (e).

There was no FABA or ASOA response from the rabbit which received antigen (a) and (b). Only the ASOA titer rose with the rabbits receiving antigen (c). Both FABA and ASOA titers rose in rabbits receiving antigens (d), (e) and (f). The FABA titer rose earlier, went higher and returned to pre-immunizing level before the ASOA titer did. The highest FABA and ASOA titers were reached with using antigen (d).

It is evident from the results of this experiment that both the FABA and ASOA are elevated in streptococcal infections but are not the same. The FABA has the characteristics of an antibody. This antibody titer rises earlier and goes higher than the ASOA titer and therefore might be a better diagnostic test for the early detection of streptococcal antibodies in sera of patients. The antigen stimulating the production of FABA has proven to be some portion of the group A streptococcal exudate other than the streptolysins.