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SMUFD D/A ltr, 14 Feb 1972

TRANSLATION NO. 1437

~~DAAA-13~~

DATE: JUN 68

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

1627

IMMUNOLOGICAL STUDIES ON CRYPTOCOCCOSIS

REPORT III. STUDIES ON THE IMMUNOLOGICAL SPECIFICITY OF THE PROTEIN FRACTION AS ANTIGEN, AND ON THE DOSES OF THE POLYSACCHARIDE FRACTION FOR IMMUNOLOGICAL DIAGNOSIS

Japanese J. Bacteriology, Vol XV,
No 7, 1960, pages 707-710

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The author reported in report I, about the extraction and purification method of the polysaccharide fraction of Cryptococcus neoformans (Cr. neof.) no. 2617 strain. In that report, it was described that a variation of the precipitin reaction of the Cr. neof. immunized rabbit changed, parallel to that of its skin reaction.

In report II, it was described that the existence of the anamnestic immunological reaction was proved by re-immunization of the Cr. neof. extracted polysaccharide fraction at a period when the once elevated antibody titer became lower and its skin reaction became negative following the initial immunization of rabbit by Cr. neof. Also, it described some cross immunological reaction among Cr. neof. and similar mycoses in which the Cr. neof. extracted polysaccharide was utilized for precipitin reaction was recognized. Judging from the results of the experiment, the polysaccharide fraction extracted from Cr. neof. no. 2617 strain is considered to be a highly specific antigen to experimental cryptococcis of the rabbit. Therefore, it was flet that the skin reaction and precipitin reaction in which the polysaccharide fraction is utilized as the antigen, can also be utilized for the purpose of immunological diagnosis of cryptococcosis.

Furthermore, the author extracted the protein fraction from Cr. neof. no. 2617 strain and compared its antigenicity against the cryptococcosis of the rabbit with that of the polysaccharide fraction.

Since the author did not have the opportunity to apply the polysaccharide fraction in a clinical case yet, it was tried on non-cryptococcosis cases and healthy subjects. The precipitin and skin reaction

were tested in these subjects for the purpose of finding the proper dosage for immunological diagnosis.

METHOD AND RESULTS:

I. Extraction method of the protein fraction. Summary of method is explained in table 1.

Cr. neof. no. 2617 strain was cultivated on 4% dextrose Sabrand's liquid media at 37°C for 72 hours with occasional stirring. After cultivation, it was centrifuged to collect the organism. The collected organism was dried after being washed three times with physiological saline and three times with acetone. Five grams of the dried organism was diluted with a triple-fold dose of physiological saline. Furthermore, the organism-body was destroyed with a homogenizer, followed by sonic wave treatment for sixty minutes. After the sonic wave treatment, it was centrifuged again. The supernatant obtained was filtered and diluted with triple-fold solution of 60-70 percent ethanol. This ethanol solution was left in the refrigerator for 24 hours, and a white sediment was obtained. Again, the sediment was dissolved in distilled water and the pH was adjusted to 3.5 with addition of 1N-HCl. After the solution remained in the refrigerator for 24 hours the second time, the sediment obtained was washed with acetone and dried. Finally, after being dried, 15 mg. of the whitish-gray powder was obtained. Biuret, ninhydrin and millon reaction of 0.1 percent solution of the powder were all positive whereas its molish reaction was pseudopositive.

II. Sensitivity of the extracted protein fraction as a diagnostic antigen against the crypotococcus immunized rabbit.

Male rabbits, weighing about two kgs, Cr. neof. no. 2617 and Tasaka strains were used for the experiment. Cr. neof. no. 2617 strain is the strain from which the polysaccharide fraction was extracted, whereas, Cr. neof. Tasaka strain is the strain isolated from a cryptococcosis patient under the case of Dr. Ueda's Internal Medicine Department, Tokyo University Hospital. The organism, after being cultivated at 37°C for 48 hours on 3 percent dextrose Sabrand's agar-agar slant media, was diluted with physiological saline to contain 1mg./ml. Each ml. of the obtained diluent after being sterilized by heat for thirty minutes at 60°C, was innoculated into the rabbit ear vein three times a week for eight weeks. Thereafter, the same dose of the live vaccine solution was injected into the innoculated rabbit a few times. During the experiment, blood was sampled frequently. The precipitin and skin reactions in which both polysaccharide and protein fraction were used as the antigen, were tested in the blood samples obtained from the innoculated rabbits. As explained in Illustrations 1 and 2, sensitivity of both

the precipitin and skin reaction tested by the protein fraction was less than that tested by the polysaccharide fraction.

III. Investigation of the doses of the polysaccharide fraction for the purpose of immunological diagnosis.

Judging from the results described in reports I and II, polysaccharide fraction was thought to be applicable clinically in the immunological diagnosis of cryptococcosis. Since the author did not have an opportunity to try either the polysaccharide or protein fraction in a clinical case, the application of these media was limited to either healthy or non-cryptococcosis patients in order to determine the proper dose of the media without causing non-specific untoward reactions. Among the non-cryptococcosis patients, psychiatric patients had a tendency to develop asymptomatic mycoses infection including Cr. neof. as the nature of the disease. All of the patients selected were adults.

A. Skin reaction

Twenty-four hours after 0.1 ml. of the polysaccharide fraction diluted to different concentrations was injected intradermally on the ulnar aspect of the forearm, the degree and duration of redness was measured. Redness of 10 mm. or larger in diameter was designated as positive; 9-5 mm. was considered to be pseudo-positive and redness 4 mm. or less in size was regarded as negative. No duration of redness developed in any of the cases.

Group I-A: Thirty eight patients, including progressive paralysis, cerebral syphilis and patients whose syphilitic serum was positive. (Patients were admitted at Yamada Hospital, Psychiatry Department, Ono Hospital, Psychiatric Department, Hachioji, Metropolitan Komagome Hospital, Dermatology Department).

Fifty mcg./ml. of the polysaccharide fraction solution were given to these patients as antigens. When 50 mcg./ml/ (5 mcg. of antigen dose) solution was used, seven of them (18 percent) were positive and six (15 percent) were pseudo-positive, while twenty five (67 percent) were negative to the skin reaction. Whereas, when 10 mcg./ml. (1 mcg. of antigen dose) solution was used, 100 percent of them turned out to be negative.

Group I-B: Eighty patients, including schizophrenia, alcoholism (admitted to Ono Hospital, Psychiatry Department, Hachio-ji).

Either 20 mcg./ml. or 10 mcg./ml. of the polysaccharide fraction solution was given to these patients, and the results proved to be negative.

Group II: Sixteen healthy subjects were selected and were given 20 mcg./ml. or 10 mcg./ml. of the polysaccharide fraction solution. No positive skin reactions were reported in this group.

Judging from the results of the skin reaction experiment, some of them manifested positive skin reaction when 0.1 ml. of 50 mcg. (1 ml.) of the solution which contained 5 mcg. of antigen dose, was given intradermally. However, it was considered a non-specific reaction since no positive or pseudopositive skin reaction was seen in those who received an antigen dose of 2 mcg. or less.

B. Precipitin reaction:

Precipitin reaction was tested with different concentrations of the antigen.

1. Precipitin reaction for those eight patients whose symphilitic serum reactions were positive, was tested by an antigen of two different concentrations. Among them, two patients (25%) manifested positive reactions, while six (75%) were negative when 1000 mcg./ml. of the polysaccharide fraction solution was given to them as an antigen. On the other hand, all eight (100%) proved to be negative when 600 mcg./ml. was given.

2. Of the eighty-four patients whose syphilitic serum reaction was negative, four (4.7%) were positive, while the remaining eighty (95.3%) were negative when 1000 mcg./ml. of the antigen solution was given. On the other hand, all eighty-four (100%) proved to be negative when 500 mcg./ml. solution was given.

As explained in the above results, no positive precipitin reaction was obtained when the concentration of the antigen solution of 500 mcg./ml. or less was used.

DISCUSSION

In the immunological diagnostic method of mycoses, the polysaccharide fraction has been most widely used as an antigen for the precipitin, skin of complement fixation test. (Martin ((3)), Lamb ((4)), and Evans ((5)). Generally, the protein fraction is difficult to extract, thermobile, relatively insoluble in water and inconvenient for experimental use.

Nevertheless, Pack and others (6) reported that antigenicity of the protein fraction extracted from Blastomyces dermatitides was just as good as that of its polysaccharide fraction, and a positive skin reaction tested by its protein fraction was found in a fair number of Blastomycosis patients. Also, Salvin and others (7) reported that better results

of skin reaction were obtained with the application of protein carbonhydrate complex which was extracted from Histoplasma capsulation than those of histoplasmin when it was given to histoplasmosis patients. Inoue (8) reported that sensitivity of the protein fraction as an antigen was slightly inferior to the polysaccharide fraction at a lower concentration.

It was concluded from the results of our experiments that sensitivity of the protein fraction extracted from Cr. neoformans (as an antigen) was inferior to the polysaccharide fraction in both precipitin and skin reaction. Frankly speaking, it cannot be denied that the protein fraction extracted in our experiment may contain a small amount of polysaccharide or other substances.

Considering such shortcomings of protein fraction as a poor sensitivity or instability, it seems that the polysaccharide fraction is the best choise of an antigen for immunological diagnosis of mycoses.

In our series of healthy and non-cryptococcosis cases, not a single patient manifested a positive cryptococcus immunological reaction when the skin reaction was tested by the polysachharide fraction antigen dose of 2 mcg. or less, and when its precipitin reaction was tested by an antigen concentration of 500 mcg./ml. or less.

I am looking forward to the opportunity of studying the polysaccharide fraction antigen in clinical cryptococcosis to evaluate its immunological diagnostic usefulness.

CONCLUSION

1. It was found that the sensitivity of the protein fraction extracted from Cryptococcus neoformans as an antigen was inferior to that of the polysaccharide fraction as far as the precipitin or skin reaction was concerned.

2. Not a single non-cryptococcosis patient demonstrated a positive reaction when their skin reactions were tested by the antigen, polysaccharide fraction of 2 mcg. or less in concentration and their precipitin reactions were examined with the antigen, of 500 mcg./ml. or less in concentration. So far, the investigation was not extended to clinical cryptococcosis. However, should a clinical cryptococcosis patient reveal positive skin or precipitin reaction in which the polysaccharide fraction is used as its antigen at an identical concentration as examined in non-clinical groups, it should be considered to be a valuable immunological diagnostic tool for crypotococcosis.

The author sincerely appreciates and acknowledges the guidance of Prof. T. Akiba and Asst. Prof. K. Iwata. His thanks are extended to the members of Tokyo University Hospital Central Laboratory, Yamada Hospital Psychiatry Department, Hachioji-Ono Hospital Psychiatry Department and the Metropolitan Komagome Hospital, Dermatology Department.

A summary of this paper was presented at the Third General Meeting of the Japan Mycology Association.

TABLE 1

EXTRACTION METHOD OF PROTEIN FRACTION OF CR. NEOFORMANS

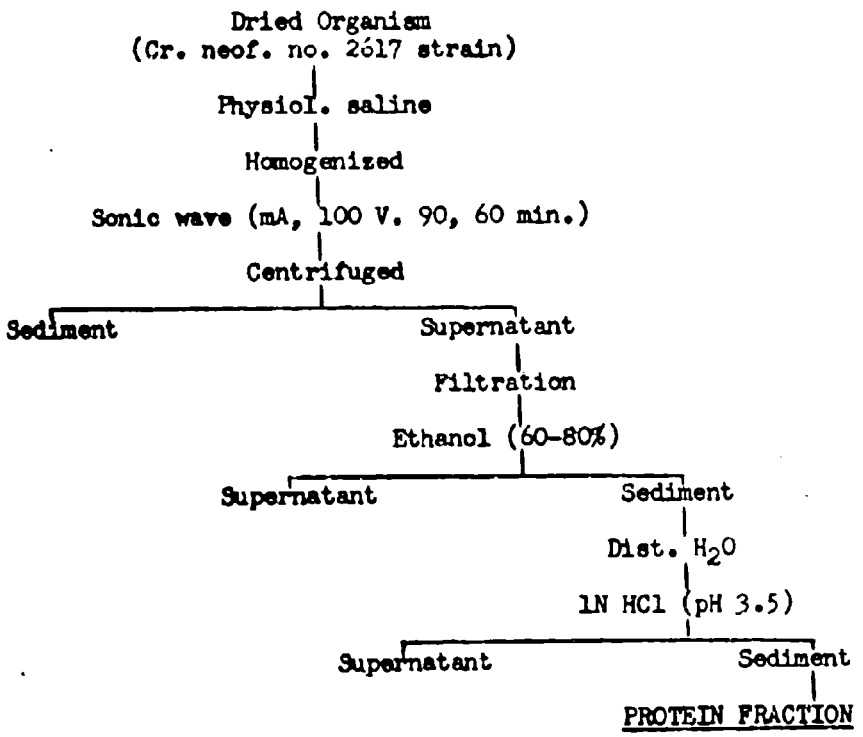

TABLE 2

SKIN AND PRECIPITIN REACTIONS OF HEALTHY AND NON-CRYPTOCOCCOSIS PATIENTS TESTED BY THE CR. NEOF. EXTRACTED POLYSACCHARIDE FRACTION

I.

SKIN REACTION

No. of Cases			50 mcg./ml.			20 mcg./ml.	10 mcg./ml.
			+	±	-	-	-
Group I	A	38	7 (18%)	6 (15%)	25 (67%)		38 (100%)
	B	80				80 (100%)	80 (100%)
Group II		16				16 (100%)	16 (100%)

Comment: Group I-A: 38 cases, including progressive paralysis, cerebral syphilis and those whose syphilitic reaction was positive.
Group I-B: 80 cases, including schizophrenia and alcoholism.
Group II: 16 cases, healthy.

II.

PRECIPITIN REACTION

(Those whose syphilitic reaction is either positive or negative)

No. of Cases		100 mcg./ml.		500 mcg./ml.
92		+	-	-
Positive	8	2 (25%)	6 (75%)	8 (100%)
Negative	84	4 (4.7%)	80 (95.3%)	80 (100%)

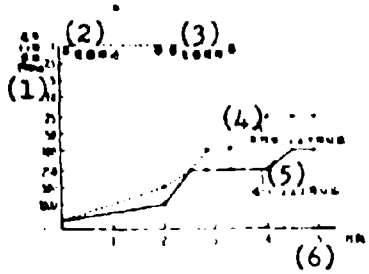

Illustration 1. Comparison of precipitin reaction of the Cr. neoformans No. 2617 strain-immunized rabbit tested by protein fraction with the one tested by polysaccharide.

Key: 1 -- Protein or polysac. fraction (mcg./ml.); 2 -- Dead vacc.; 3 -- Live vacc.; 4 -- Precipitin fraction tested by polysacc. fraction; 5 -- Precipitin reaction tested by protein fraction; 6 -- no. of months.

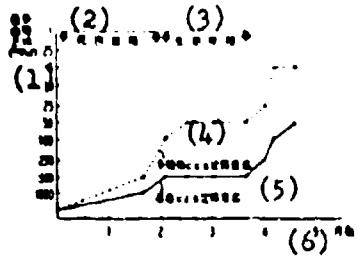

Illustration 2. Comparison of Cr. Neof. Iasaka strain-immunized rabbit tested by protein fraction to that tested by polysaccharide fraction.

Key: (Same as for Illustration 1)

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