Reports

Immunization against Trypanosoma lewisi in Rats by Injections of Metabolic Products

The early cessation of reproduction that is characteristic of *Trypanosoma lewisi* infections in rats was recognized by Taliaferro (*1*) to be due to an antibody, ablastin, which he and others considered distinct from the trypanocidal antibodies that are believed to be responsible for the number crisis and for ultimate eradication. Subsequently a third, agglutinating, antibody was postulated (*2*). Serum containing ablastin, passively transferred, inhibits reproduction and is therefore protective. It is not absorbed by the parasites, does not sensitize them, and does not physically injure them, for they reproduce normally when washed and transferred to a nonimmune host.

We felt that ablastin might well be an antibody directed against metabolic products (secretions or excretions or both) of the parasites, as one of us (*3*) previously suggested. To test this hypothesis, we attempted to immunize rats by injection of trypanosome-free metabolic products. Blood containing numerous dividing trypanosomes was obtained by aseptic heart puncture of rats before any demonstrable ablastin had formed. The trypanosomes were separated by centrifugation, washed with saline, suspended in equal parts of normal rat serum and saline so that there were about 200 million parasites per milliliter of medium, and incubated for 24 hours at about 27.5°C. With addition of 0.0025 g of glucose per milliliter after about 12 hours, they remained actively motile for the entire 24 hours. After incubation, the majority of the trypanosomes were separated by centrifugation, and the supernatant, containing the metabolic products of the parasites, was filtered through fritted glass (fine porosity) and stored in a deep freezer until ready for use. No deformed or disintegrating trypanosomes, and very few nonmotile ones, were seen among thousands that were examined and counted after incubation, and there was no decrease in numbers; care was taken not to crush any of the parasites during filtration. The separated trypanosomes were then washed in saline and triturated by repeated freezing and thawing until test inoculations showed that no infective organisms were left.

Six 100-g rats were each given six intraperitoneal injections, at 3-day intervals, with metabolic products from a total of 4000 million trypanosomes, approximately the number present in a 100-g rat at the height of an infection, and six others were each similarly injected with the triturated bodies of the 4000 million trypanosomes from which the metabolic products had been obtained; six controls were given injections with normal serum and saline, and six others were not injected. On the tenth to 12th day after the last injection all rats were inoculated intraperitoneally with 50,000 washed adult trypanosomes, after which daily blood examinations were made.

All the rats in the control groups had typical infections reaching peaks of from about 200,000 to 600,000 trypanosomes per cubic millimeter. Of the rats injected with trypanosome bodies, all became positive in the normal time; five had low peaks of from less than 500 to 7500/mm^3 and became negative after the fifth to 11th days, and one reached a peak of 200,000/mm^3 for 1 day and became negative after the 16th day. The six rats injected with metabolic products remained completely negative throughout.

Six additional rats were given six injections with the metabolic products of a total of 2000 million trypanosomes and, with three controls, were inoculated with 20 million trypanosomes 10 days after the last injection. In the control rats, the blood became positive within 1 hour, and the infections ran a normal course, but in the injected rats again there was complete protection, since no trypanosomes were ever seen in the blood smears.

Using welled slides, we added adult trypanosomes to a few drops of serum from each of the following: (i) normal rats; (ii) rats that had been "immunized" with triturated trypanosome bodies; (iii) rats that had been "immunized" with metabolic products; and (iv) rats that had recovered (the serum was taken 6 weeks after infection). Observations were made at intervals of a few minutes to 1 hour for 6 hours. The parasites in the serum from group i, diluted 1/3, remained active and unagglutinated throughout. Those in the serum from group ii, diluted 1/3, showed a slight degree of agglutination at 15 and 30 minutes, but by 45 minutes the parasites in the few small clumps were dispersing, and after 1 hour they were unagglutinated and normal in appearance and motility. In contrast, in the serum from group iii, agglutination began within 5 minutes in serums diluted 1/1 to 1/5, and in 10 minutes in serums diluted 1/10, 1/25, and 1/100. Up to a dilution of 1/5, practically all the parasites became agglutinated in large clumps within 10 or 15 minutes, and in dilutions up to 1/100 somewhat later. Slight agglutination occurred at a dilution of 1/200, but none at 1/400 or above. In all cases agglutination was accompanied by a marked loss of swimming activity, but motility continued. In the serum from group iv, the results were identical with those in group iii at dilutions up to 1/10, but at 1/100 the effect was somewhat weaker; the agglutination was not as complete or in as large clumps. In a test with serum from group iii collected 6½ months after the last immunizing injection, there was some loss of titer, but the 1/1 serum had lost none of its potency.

Preliminary absorption experiments with immune serum from recovered rats indicate that absorption with lyophilized metabolic products markedly reduces the protective power of the serum and permits practically normal reproduction when administered to rats at the rate of 1 ml/100 g 1 hour prior to intraperitoneal injection of 20 million adult trypanosomes. In contrast, immune serum absorbed with trypanosome body substance loses little if any of its protective power and, as when unabsorbed serum is used, no dividing forms are found. This indicates that the antimetabolic-products antibody must be regarded as ablastin, even though the rapid destruction of trypanosomes in the presence of adequate amounts of the antibody has prevented direct demonstration of inhibition of reproduction.

Our results indicate that agglutination and reduced locomotion, some of the effects hitherto attributed to trypanocidal or other antibodies (*2*, *4*), and which render the parasites more susceptible to being filtered out of the blood and phagocytized, are really produced by ablastin.

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These phenomena may be the result of antibody combination with excreted metabolic products adhering to the surface of the trypanosome bodies. Further work on the protective power of antimetabolic-products serum, with or without absorption, is in progress.

It is probable that ablastin acts specifically against an enzyme or enzymes concerned in the nutrition of the parasites, as was postulated by one of us (*3, 5*) when the similarity between inhibition of reproduction of *Trypanosoma lewisi* and the interference with growth, development, and reproduction of the nematode *Nippostrongylus muris* was pointed out.

We believe that this production of functional immunity through the agency of antibodies directed against metabolic products, which interferes with nutrition, will be found to be a widespread phenomenon among infectious organisms (*6*).

CARLOS J. THILLET, JR.*
ASA C. CHANDLER
Rice Institute, Houston, Texas

References and Notes

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6. This work was supported by a research grant from the National Institutes of Health.
* Present address: University of Puerto Rico Medical School, San Juan, Puerto Rico

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Chromatographic Microassay for Cholesterol and Cholesterol Esters

Free cholesterol is readily separated from its esters by column chromatography on silicic acid. The separation is quantitative, and the two components can then be assayed separately. The procedure described here (*1*) requires relatively small samples and can be completed in less than 1 hour. With a simple modification of the extraction procedure, it is possible to get a reliable assay using only 0.02 ml of human plasma or 0.05 ml of rat plasma. The 100-mesh silicic acid sold by Malinckrodt especially for chromatography is satisfactory for this purpose. Ordinary reagent-grade products may be activated by heating them at 120°C overnight (*2*) and then quickly sieving them once in a moist atmosphere. The activated silicic acid must be protected from atmospheric moisture.

For quantitative assay, we use a chromatographic tube made of 8-mm (outside diameter) glass tubing about 25 cm long. The tube is abruptly constricted on one end to a diameter of 2 to 3 mm inside, and the end is drawn out about 10 mm to form a short nipple. To prepare the column, ram a small pledget of cotton down the tube to plug the constricted end. Fill the tube to a height of about 5 cm with silicic acid by means of an eye dropper and pack it by applying air at a pressure of 7 to 10 lb/in.^{2} to the top of the column. Prewet the column by forcing through about 2 ml of petroleum ether by means of the same air pressure.

For the routine assay of human plasma or serum, our procedure is as follows. Mix 0.1 ml of plasma or serum with 5 to 7 ml of 1/1 methanol and acetone. Bring the mixture to boiling, cool, dilute to 10 ml with solvent, and filter. Evaporate a 3.0-ml aliquot by placing the tube in cold water and bringing the water to boiling. Invert the tube while it is cooling to drain out the residual vapor of the solvent. Transfer the lipid to the column with three 2-ml portions of petroleum ether (boiling range 30° to 60°C) by means of an eye dropper.

Rinse the test tube and the chromatographic tube with each portion of solvent and force each through in turn. The flow rate should never be greater than 4 ml/min with any of the solvents. Care should also be taken never to allow the solvent level to go below the top of the silicic acid in this step and in the next one. At this point the free and esterified cholesterol are adsorbed at the top of the column. (This may be demonstrated by extruding the column by means of air pressure gently applied to the bottom of the tube and streaking it with concentrated sulfuric acid).

Develop the column with about 4 ml of 1/1 chloroform and petroleum ether. Collect this eluate, which contains the esterified cholesterol, in a separate test tube. Rinse the outside of the tip of the chromatographic tube with about 1 ml of petroleum ether, allowing the ether to run into the same test tube. To remove the free cholesterol from the column, elute with 4 ml of 1/1 methyl or ethyl acetate and petroleum ether. Force this through until flow ceases, and rinse the tip as before. Extrude the column by means of air pressure as described in the preceding paragraph and discard the silicic acid.

Evaporate the solvents in the aforementioned manner and take the fractions up in 3.0 ml of glacial acetic acid. Warm the tubes containing the esters to effect solution. Cool and add 2.0 ml of the ferric chloride-sulfuric acid reagent of Brown *et al.* (*3*) and mix thoroughly. Read the optical density at 560 mμ in the Coleman universal spectrophotometer. For precise work, it is necessary to prepare chromatographed solvent blanks. The standard contains 50 μg of cholesterol in 3.0 ml of acetic acid, and the blank for this is not chromatographed. The color develops rapidly and is stable for hours. With rat plasma or serum, an 8-ml aliquot of the methanol-acetone extract is required for a satisfactory reading of optical density.

Table 1. Estimation of free and stearyl cholesterol in mixtures containing different amounts.

Mixture of free and stearyl cholesterol chromatographed (mg of each)	Stearate found		Free cholesterol found	
	(mg)	(%)	(mg)	(%)
2.00	2.07	103.5	2.07	103.5
0.200	0.207	103.5	0.201	100.5
0.020	0.0201	100.5	0.0199	99.5

This procedure separates plasma cholesterol into its free and esterified fractions. If a plasma extract, or a mixture of cholesterol and its stearate, is placed on the column and the development with chloroform and petroleum ether is continued beyond the usual amount, it is found that the first two milliliters of eluate contain a large amount of cholesterol. This is esterified cholesterol. The third milliliter of eluate contains only a trace of cholesterol, and the fourth to about the tenth milliliters have none. As the development is continued beyond the tenth milliliter, the free cholesterol appears in the eluate. It is all eluted after about 20 ml of chloroform and petroleum ether have been used.

In Table 1 we see that the column will function satisfactorily over a very wide range of cholesterol or cholesteryl stearate concentration. In Table 2, evidence is presented that free cholesterol or its stearate added to an extract of plasma can be quantitatively demonstrated. Rat adrenal cholesterol is assayed by extracting the macerated gland in a hot mixture of methanol and ace-

Table 2. Recovery of free and of stearyl cholesterol which were added to plasma extract. The amounts added and found are given in milligrams per 100 ml of plasma.

Compound	Amount		Recovery (%)
	Added	Found	
Stearyl cholesterol	150	151	101
Free cholesterol	100	104	104