5827

Production of Anti-Sheep Hemolysin in a Donkey.

C. E. LIM AND D. HUIE WONG.

From the Department of Bacteriology and Immunology, Peiping Union Medical College, Peiping.

In the complement fixation test for the diagnosis of syphilis or other diseases, it is usual to use an anti-sheep hemolysin for the production of which rabbits have been employed almost exclusively. Numerous methods have been proposed for the immunization of these animals to produce a high titer serum, but we have found in our routine immunization of such animals that they vary considerably in their individual response to the injections of sheep erythrocytes.

For a routine diagnostic laboratory handling a large number of Wassermann tests where a constant and reliable supply of hemolysin is required, it seems desirable to immunize a larger animal which will yield a supply of hemolysin sufficient to last for a number of years. This will obviate the necessity of immunizing rabbits for this purpose at short intervals and will result in an economy in time and money. Attempts have been made to produce anti-sheep hemolysin on horses but Gilbert[1] was unable to produce it in a horse showing no natural hemolysin in the blood, but was successful with a mule having a small amount of hemolysin before immunization. As these animals are rather costly, it appears to us worth while to find a less expensive experimental animal.

Our choice has accordingly fallen on the donkey, a common beast of burden, which is relatively inexpensive, costing about 20 times the price of a rabbit here, and is of a convenient size for our purpose. Before immunization the blood of the donkey was first tested for the presence of natural anti-sheep hemolysin, but not a trace of it was present. Immunization was then begun by a series of inoculations given through the ear vein. All series of injections were given on 3 successive days at 4-day interval between each series and trial bleedings were taken each day before injection. The first 2 series of injections made with small amounts of washed sheep blood cells in 10 cc. doses of a 10% suspension produced no detectable hemolysin. The third series of injections, consisting of 20 cc. of a 10% suspension of sheep cells followed by 2 injections of the same amount of a

[1] Gilbert, R., *New York State J. Med.*, 1922, **22**, 286.

20% suspension, caused the formation of a small amount of hemolysin (titer 1:120). In the fourth series, the injection of 10 cc. of packed sheep cells caused the animal to have a severe shock, showing signs of weakness of the legs and heavy deep respiration. The titer of hemolysin on a trial bleeding taken on the next day immediately before the second injection of a similar dose increased to 1:800. After the second injection the donkey dropped on the floor and its respiration became very slow and deep. It took one-half an hour for the donkey to recover from the shock. It was, therefore, decided to allow the animal to rest for several days before re-injection. One week later, blood taken before the fifth series of injections was started showed the titer to have risen to 1:2400. The animal suffered no shock from the 3 injections of this series although the dose was increased to 15 cc., but the hemolysin titer rose to 1:4000. Since a sixth series of injections of the same amount of antigen failed to raise the titer, 2 liters of blood were bled from the jugular vein. An immediate drop of the titer to 1:2000 followed the bleeding as indicated in the trial bleeding taken the next day. Another bleeding taken 10 days later caused the titer to drop to 1:1000. The animal became thin and weak and was allowed to rest. Trial bleedings taken once a week showed that the hemolysin titer rose again to 1:2000 without any further injection and remained at this level for 4½ weeks, when another series of injections was started. Ten cc. followed by 2 injections of 15 cc. of packed sheep cells on 3 consecutive days with 4 days of rest between each series were given as before. The titer gradually rose again, reaching 1:3200 within 3 weeks. After the fourth series of such injections in this attempt at restimulation of the antibody production, the titer fell to 1:2000. This would tend to militate against further injections and 2 liters of blood were accordingly bled from the jugular vein. The titer on a trial bleeding taken the next day fell to 1:1000. It rose again to 1:1600 and remained so for 6 weeks, after which the animal was discarded.

One of the main objects in the production of hemolysin is to obtain a high titer serum and to preserve it in such a way as to retain its potency. The highest titer obtained in our experiment is 1:4000 and the average is 1:2000, which compares favorably with that found in the mule by Gilbert. Our hemolysin produced in the donkey was divided into 3 parts for preservation, one part being put up in ampoules aseptically without any preservative, one part put up with 50% neutral glycerol and the third part preserved with 0.02% mercuric chloride. The glycerol and mercuric chloride caused a slight

drop in the titer of the serum immediately after addition, but up to this time (5 months of preservation) none of the serum has showed any additional change. Specimens from all 3 methods have been used successfully in the routine Wassermann tests done in our college hospital for the last 5 months. The supply of hemolysin thus obtained is enough to last us for a number of years and the rates of deterioration in the various methods of preservation will form the subject for further study.

The results reported here suggest the conclusion that anti-sheep hemolysin of a satisfactory titer for use in the complement fixation reaction can be produced in a donkey having no natural hemolysin.

5828

Technique for the Complete Preservation of Supravital Stain of Neutral Red in Paraffin Sections.

C. H. HU. (Introduced by S. H. Liu.)

The technique for the preservation of the supravital stain of neutral red in paraffin sections has been described by McJunkin[1] and Forkner.[2] Their methods enable one to observe most of the neutral red present in the cells; but a certain amount of the stain is inevitably lost in the process of preparation, and they require rapid dehydration and strict limitation of the size of the tissue blocks. The method described below has the advantage over those previously described, in that the entire neutral red stain within the cells is faithfully preserved, that the size of the blocks can be reasonably large, and that the different steps of staining can be carried out in a more leisurely and comfortable manner.

In this technique, advantage is taken of the fact that neutral red is only slightly soluble in aqueous or alcoholic solution containing mercuric bichloride. After the minimal amount of the bichloride required in each solution for the maximal insolubility of the neutral red is determined, any solution which will check further dissolution of neutral red from the tissue can be easily prepared from it.

The tissue to be studied is best stained by the direct injection method described by Forkner with only a slight modification through replacement of his Zenker-formalin by solution A (see below).

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[1] McJunkin, F. A., *Am. J. Path.*, 1925, **1**, 305.

[2] Forkner, C. E., *J. Exp. Med.*, 1930, **52**, 379.