DETECTION OF SURFACE ANTIGEN IN *RICKETTSIA TSUTSUGAMUSHI* INFECTED MOUSE RETICULOENDOTHELIAL CELLS

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INTRODUCTION

The clinical diagnosis of scrub typhus is difficult in endemic regions because the cardinal signs, eschar and rash, are frequently absent (Brown *et al.*, 1976, 1978). Laboratory confirmation of scrub typhus is by serologic techniques, most conveniently a microimmunofluorescent antibody assay (IFA) (Robinson *et al.*, 1976). Changes in rickettsial antibody titer are not detected until the second week of disease (Bozeman and Elisberg 1967), usually too late to influence patient management. Isolation of *Rickettsial tsutsugamushi* from patient blood can take up to 3 months and is seldom attempted.

The development of a method for the early diagnosis of scrub typhus would be a great advance. In search of such a method, we investigated the presence of rickettsial antigen on the surface of reticuloendthelial (RE) cells infected with *R. tsutsugamushi*.

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In conducting the research described in this report, the investigators adhered to the 'Guide for the Care and Use of Laboratory Animals', prepared by the Committee on Care and Use of Laboratory Animal of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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MATERIALS AND METHODS

The Gilliam strain of *R. tsutsugamushi* was plaque purified, (Oaks *et al.*, 1977) quantified, propagated, and stored as previously described (Catazaro *et al.*, 1976). Inbred mice were obtained from colonies in the Department of Laboratory Animal Resources, U.S. Army Medical Research Unit, Malaysia. All mice inoculated with *R. tsutsugamushi* were subsequently maintained on chloramphenicol (2.5 mg/ml) supplemented water (Shirai *et al.*, 1979).

Two groups (A and B) of 25 CBA/CaJ mice were immunized to prepare anti-Gilliam antibody (anti-G-Ab) and anti-C57Bl/6J RE cell antibody. Donor C57Bl/6J mice were inoculated intraperitoneally (IP) with 10^3 MLD_{50} (1,000 50% mouse lethal dose) of Gilliam. Fourteen or 21 days later RE cells were obtained from the spleens and peritoneal exudate of these mice.

Group A mice received 14 day RE cells and Group B received 21 day RE cells. The CBA/CaJ mice received 7 weekly IP injections of 2×10^7 donor RE cells in 0.5 ml physiologic saline and were maintained on chloramphenicol-supplemented water throughout the experiment. Recipient mice (Groups A and B) were bled by cardiac puncture 14 days after their last immunizing injection. Serum from like groups was pooled.

The immunizing procedure was designed to simultaneously produce anti-G-Ab and alloantibodies, predominantly of anti-$H2^b$

specificity, which could be used as a measure of RE cell immunization.

Sera from donor and recipient mice were tested for the presence of anti-*R. tsutsugamushi* whole Gilliam strain antibody using intact *R. tsutsugamushi* as antigen in the IFA assay (Cherry *et al.*, 1961). The pooled recipient sera were tested for the presence of cytotoxic antibody using freshly harvested C57B1/6J RE cells and 20% absorbed guinea pig complement. Briefly, dilutions of the serum were incubated with normal C57B1/6J RE cells for 3 hr at 37°C in 5% CO_2-95% air. The number of viable cells (as determined by trypan blue exclusion) per 300 counted was determined in triplicate assays (Lim and Murphy, 1980). After confirming the presence of anti-$H2^b$ cytotoxic specificity, absorptions were done to remove all but antibody directed toward rickettsial antigens associated with Gilliam infected RE cells. The anti-$H2^b$ cytotoxic antibody was absorbed 3 times using 1×10^8 normal C57B1/6J RE cells per ml of serum. The absorbed sera were then precipitated with ammonium sulfate and conjugated with fluorescein isothiocyanate (FITC) (Cherry *et al.*, 1961).

Specimens for examination were prepared by harvesting RE cells from Balb/cyJ mice inoculated IP with 10^3 MLD_{50} Gilliam 2, 7, 14 or 21 days earlier. The RE cells were adjusted to 2×10^5 cells per ml. As a control for rickettsial strain specificity RE cells were harvested from Balb/cyJ mice inoculated IP with 10^3 MLD_{50} of the Karp strain of *R. tsutsugamushi* 14 or 21 days earlier. Spot slides of RE cells were fixed with methanol and stained with Giemsa to assess the intracellular rickettsial burden. For examination of fluorescent antibody, three 5 µl droplets of the cell suspension were placed on a glass slide, air dried and fixed with acetone for 10 min. The FITC conjugate was applied to each cell spot and examined under a fluorescence microscope (Leitz Ortholux) as described by Robinson *et al.* (1976). Normal Balb/cyJ and C57B1/6J RE cells were used as negative RE cell controls. An FITC conjugate prepared from normal CBA/CaJ serum was used as a negative control for FITC conjugate staining.

RESULTS

The anti-*R. tsutsugamushi* whole Gilliam strain antibody titers in donor and recipient mice are shown in Table 1. The anti-C57B1/6J (anti-$H2^b$) cytotoxic activity of the CBA/CaJ pooled sera before absorption is shown in Table 2. Absorption of sera from both Groups A and B with normal C57B1/6J RE cells resulted in more than 95 per cent removal of cytotoxic activity.

Table 1

Specific anti-*R. tsutsugamushi* whole Gilliam strain antibody titers in Gilliam infected C57B1/6J donors of reticuloendothelial cells and CBA/CaJ recipients measured by the indirect fluorescent antibody (IFA) assay.

A. Geometric mean titers of donor mice

Mouse Strain	Days after Gilliam infection	
	14	21
C57B1/6J	12.50	139.78

B. Geometric mean titers of recipient mice

Mouse Strain	Days after Gilliam infection in donor mice	
	14	21
CBA/CaJ*	86.24	16.93

*CBA/CaJ mice received 7 weekly immunizing injections of 2×10^7 reticuloendothelial cells from infected C57B1/6J donor mice.

Table 2

Anti-C57Bl/6J cytotoxic antibody in pooled sera from CBA/CaJ mice immunized* with reticuloendothelial cells from C57Bl/6J mice infected with *R. tsutsugamushi* Gilliam strain.

Dilution of serum	Days after Gilliam infection in donor mice	
	14	21
1:2	87†	84
1:4	79	79
1:8	50	49
1:16	24	29

*CBA/CaJ mice received 7 weekly immunizing injections of 2×10^7 reticuloendothelial cells from infected C57Bl/6J donor mice.

†Percent mortality in fresh C57Bl/6J RE target cells.

Note. Normal mouse serum plus complement caused 9% reticuloendothelial cell mortality, and normal mouse serum alone caused 3% reticuloendothelial cell mortality in the assay.

Examination of Giemsa stained slides from the Gilliam infected Balb/cyJ mice reveled few rickettsia on day 2 and less on days 7, 14 and 21, presumably because rickettsial proliferation was controlled in the mice receiving chloramphenicol supplemented drinking water.

FITC conjugate anti-G-Ab produced from Group A mice bound to Gilliam rickettsiae in Balb/cyJ RE cells infected 2, 7 or 14 days earlier (Fig. 1), but did not bind to the surface of any RE cells examined on those days. The surface of 21 day RE cells, however, stained very faintly with the Group A conjugated antiserum. In contrast, using the same RE cell preparations, anti-G-Ab from Group B mice caused a definite surface fluorescence on RE cells harvested 21 days after infection (Fig. 2). However, this antiserum stained rickettsiae in the RE cell preparations only faintly. The surfaces of normal Balb/cyJ and CBA/CaJ RE cells were not stained with

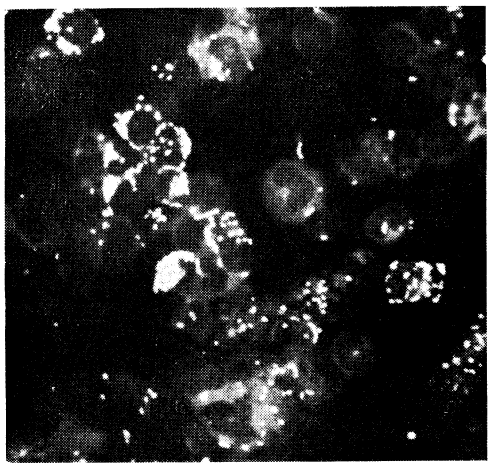

Fig. 1—Fluorescein isothiocynate conjugated Group A serum binding to reticuloendothelial cells from Balb/CyJ mice infected with Gilliam strain *R. tsutsugamushi* 2 days previously (400x)

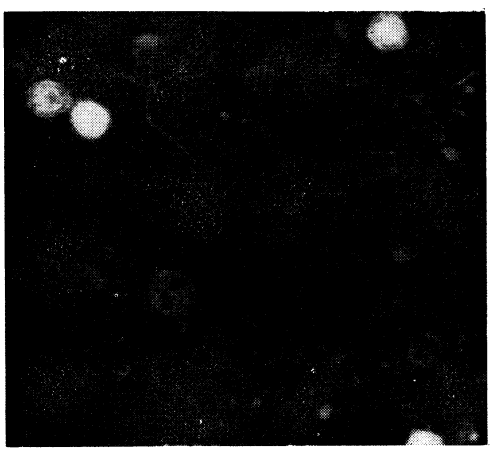

Fig. 2—Fluorescein isothiocynate conjugated Group B serum binding to reticuloendothelial cells of Balb/CyJ mouse infected with Gilliam strain *R. tsutsugamushi* 21 days previously. (400x)

Group A or B antisera. Similarly, there was no surface staining of Karp infected Balb/cyJ RE cells.

DISCUSSION

The CBA/CaJ mice immunized with Gilliam infected C57Bl/6J RE cells produced antibody of different specificity depending on whether

the immunizing cells had been infected 14 or 21 days earlier. The Group A mice which received more recently infected RE cells produced antibody with an anti-whole Gilliam titer of 86. This FITC conjugated Group A autiserum pool stained Gilliam rickettsiae in infected Balb/cyJ RE cells. The Group B mice had an anti-whole Gilliam antibody titer of only 17 and failed to stain the whole rickettsiae. This FITC conjugated Group B antiserum pool produced surface staining of 21 day post infection Balb/cyJ RE cells.

The antigen recognized by the Group B antiserum was presumably associated with the immunizing C57B1/6J RE cells. The source of the antigen recognized on infected Balb/cyJ RE cells could be the interior of the cell, with incorporation into the cell membrane, or the antigen could be rickettsial proteins circulating in the infected host which absorbed onto the cell surface. Rickettsial units assemble in the cytoplasm of infected cells (Hase, 1983) and in the late stages of *in vitro* infection, cells have been shown by scanning electron microscopy to be covered with outbudding rickettsiae (Tsuruhara *et al.*, 1982). Cells infected with *R. tsutsugamushi* have been shown to elaborate a cell surface associated neoantigen which is dependent on host cell metabolism (Dr. Thomas Jerrells, pers. commun.). Therefore, it is likely that the majority of conjugated anti-G-Ab staining observed in our experiments was associated with the host cell surface.

We cannot explain why surface staining could not be detected earlier than 21 days after infection but the reduced numbers of rickettsiae present, because of chloramphenicol treatment, may have played a part. The demonstration of rickettsial antigen on RE cells provides a possible method for early diagnosis of *R. tsutsugamushi* infection. The use of conjugates such as that prepared for the Group B mice may be feasible for an assay, if a higher titered conjugate can be produced.

SUMMARY

Antibody produced by immunizing CBA/CaJ mice with RE cells from C57B1/6J mice infected 14 days earlier with *R. tsutsugamushi* Gilliam strain bound readily to Gilliam strain non-cell associated rickettsiae and less readily to the periphery of infected RE cells. Conversely, antibody produced by immunizing with RE cells infected 21 days earlier did not bind to Gilliam rickettsiae but bound to the surface of RE cells from mice infected 21 days earlier. This binding was not related to alloantibodies because these were absorbed prior to testing.

The demonstration of rickettsial antibody staining of infected cell associated antigen(s) in this assay system provides a new method for the detection of *R. tsutsugamushi* infection.

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