The C3 titre of sera from rats pretreated with endotoxin showed in vitro a marked decrease on addition of the lower endotoxin dose and practically disappeared on addition of the higher dose. In contrast, in vivo even 1.0 ml/100 g of endotoxin failed to reduce the C3 level.

SNYDERMAN et al.[8] and LICHTENSTEIN et al.[9] showed that the titre decrease of excusively of C3 complement component in vitro is associated with formation of those biologically active substances – anaphylatoxin, chemotactic substances – which may play a role in the development of endotoxin shock. Our experiments indicate that in rats prepared with endotoxin, provocation fails to activate the full complement system and accordingly neither anaphylatoxins, nor chemotactic substances are formed.

Conclusions as to the nature of the mechanism responsible for the inactivity of the provoking endotoxin would be premature.

Based on the above findings, the C3 reducing action of endotoxin may be utilized for the study of the mechanism of endotoxin tolerance induced by serial treatment.

Résumé. L'effet d'une dose létale d'endotoxine, qui abaisse in vivo le taux du C3 du rat ne se manifeste pas chez les rats rendus tolérants à cette substance.

G. FÜST and GABRIELLA FÓRIS

National Institute of Public Health, and State Institute of Pharmacy, Budapest (Hungary), 8 April 1970.

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Antigenic Components of Normal and Burned Mouse Skin

The presence of specific newly formed toxic antigens in burned tissues has been suggested by several authors[1–4]. Other investigators have failed to demonstrate either antigens or antibodies which uniquely arise following thermal injury[5,6]. Additional studies showed that both burned and normal skin extracted under sterile conditions yielded substances toxic in either normal or burned mice[7–9]. The present study was undertaken to determine if there were differences in the antigenic components in normal and burned mouse skin.

Material and method. Female CF-1 mice (Carworth Farms) weighing 22–24 g were shaven and anesthetized with penthrane. An asbestos board with 1×1.5 inch window was pressed firmly against the shaven back. Ethanol (0.5 ml) was evenly spread over the area of the back outlined by the window, ignited and allowed to burn for 15 sec. This caused a burn of approximately 35% of the body surface. 10–15 min post burn, the mice were sacrificed and the burned skin excized. The skin tissue was ground with saline and sand (0.75 g) using a mortar and pestle; the ratio of tissue to saline being 4.5 g/15 ml. After 10 min maceration, the ground tissue was passed through several layers of gauze to remove large particles and was then centrifuged at 15,000 *g*/60 min in a refrigerated International Model B 20 centrifuge. The pellet and floating lipid layer were discarded and the burned tissue extract was filtered through a 0.45 μ Millipore filter and refrigerated. The same procedures were used with normal mouse skin to prepare normal tissue extract. These materials were used as antigens for double diffusion gel precipitation tests.

To obtain a greater quantity of antigen, the extraction procedure was modified and 30 g of burned skin were homogenized in a Waring blender (Model 1042) for 10 min at highest speed. This procedure was carried out at 4 °C. At this point, the extract was treated in an identical manner to that described above. Similar procedures were used to prepare normal tissue extracts. While the total protein values per ml of extract prepared in this manner was 3 times higher than those prepared using hand grinding, the ratio of protein was similar in both methods.

Rabbits were immunized as follows: Lyophilized normal or burned tissue extract which contained 18 g of protein was emulsified in 2.5 ml of saline plus an equal volume of complete Freund's adjuvant. Groups of 6 Dutch Belted rabbits, 300–500 g, were inoculated s.c. and i.m. in several places. After a 45 day resting period the respective groups of rabbits were given 2–3 ml of burned or normal tissue extract s.c. and i.m. in 0.5 ml amounts and the rabbits were bled 10 days later. The antisera obtained from the 6 rabbits were pooled and used in gel diffusion studies.

To adsorb out antibodies to mouse serum from the rabbit serum, 0.1 ml of normal mouse serum was added to 2 ml of antisera, incubated at 37 °C for 1 h, refrigerated overnight and any precipitate formed removed by centrifugation. This procedure was repeated until no further precipitation occurred. Similarly 2 ml of tissue extracts were treated with 0.1 ml of goat anti-mouse serum and treated as outlined above. This procedure removed the normal mouse serum components from the tissue extracts.

The antiserum treated as outlined above was then subjected to ammonium sulfate fractionation to obtain γ-globulin[10] and the γ-globulin was concentrated 4-fold.

Double diffusion gel precipitation tests were performed in petri dishes using 1% agarose in borate-buffered saline at pH 7.8. Wells were filled once every 24 h for 3 days, incubated 48 h and then filled once every 24 h for 3 more days. Incubation was done at room temperature. Refilling the wells in this manner gave the best results of the various filling procedures tested.

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To obtain post-burn serum, mice were burned as previously described and blood drawn by capillary tubes inserted in the retro-orbital sinus. Samples of blood were obtained at 15, 30 and 60 min postburn. The blood was allowed to clot and the serum separated from the clot by centrifugation in a micro hematocrit centrifuge. Sera from several mice were pooled.

Since serum from patients recovering from burns had been proposed as a therapeutic agent in thermal injury[1,2] we felt that it was important to determine whether any antibodies to skin antigens were contained in convalescent mouse serum. Such serum was obtained from animals burned for 11 sec only, to insure a greater number of survivors. These mice were bled by cardiac puncture 14 days later.

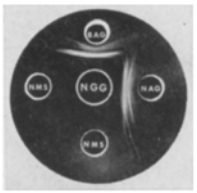

Fig. 1. Double diffusion gel precipitation test. Central well: Concentrated γ-globulin from antiserum prepared by immunizing rabbits with normal mouse skin extracts (NGG). Peripheral wells, clockwise from the top well: Burned mouse skin extract (BAG); normal mouse skin extract (NAG); normal mouse serum (NMS).

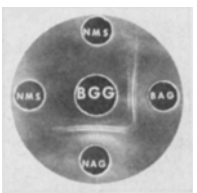

Fig. 2. Double diffusion gel precipitation test. Central well: Concentrated γ-globulin from antiserum prepared by immunizing rabbits with burned mouse skin extracts (BGG). Peripheral wells, clockwise from the top well: Normal mouse serum (NMS); burned mouse skin extract (BAG); normal mouse skin extract (NMS).

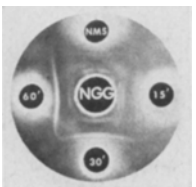

Fig. 3. Double diffusion analysis of skin antigens demonstrated in the serum of burned mice at various times after thermal trauma was applied. Central well: Concentrated γ-globulin prepared by immunizing rabbits with normal mouse skin extracts (NGG). Peripheral wells, clockwise from top: Normal mouse serum (NMS); serum obtained from mice 15, 30 and 60 min after thermal injury.

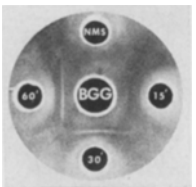

Fig. 4. Double diffusion analysis of skin antigens demonstrated in the serum of burned mice at various times after thermal trauma was applied. Central well: Concentrated γ-globulin prepared by immunizing rabbits with burned mouse skin extracts (BGG). Peripheral wells, clockwise from top: Normal mouse serum (NMS); serum obtained from mice 15, 30 and 60 min after thermal injury.

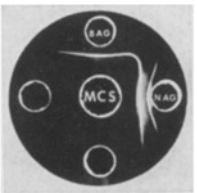

Fig. 5. Double diffusion analysis of anti-skin antibodies in serum of mice 14 days after thermal injury. Center well: 14 day convalescent serum obtained from burned mice (MCS). Peripheral wells, clockwise from top: Burned mouse skin extract (BAG); normal mouse skin extract (NAG); the two additional wells remained empty.

The Ouchterlony plate in Figure 1 shows that when γ-globulin prepared from antisera made by immunizing rabbits with normal mouse skin extracts (NGG) was tested against normal (NAG) and burned (BAG) mouse skin extracts there appeared to be similar numbers of precipitin lines formed with each antigenic preparation. In addition, these lines seemed to be identical in the two preparations. Similar results (Figure 2) were obtained when γ-globulin prepared from antiserum made by immunizing rabbits with burned mouse skin extracts (BGG) was used. The γ-globulin obtained from the antiserum against normal skin antigens (Figure 1) gave a stronger reaction. The control wells filled with normal mouse serum showed that the γ-globulin preparations were free of antibody which would react with mouse serum components.

In order to determine whether any skin antigens entered the circulation after thermal trauma, mouse serum obtained at various times post-burn was reacted with the γ-globulin preparations. Figures 3 and 4 show that by 30 min after the burn was applied, skin antigens could be demonstrated in the serum of the burned animals. Here again, the γ-globulin prepared from antiserum against normal skin tissue (Figure 3) appeared to be more effective in demonstrating the presence of the antigens.

When 14 day post-burn convalescent serum from mice (MCS) was used as the anti-skin antiserum (Figure 5) precipitin bands were formed against antigens found in both normal and burned skin antigenic preparations. It would, therefore, seem apparent that after thermal injury autoantibodies were formed to these antigens.

These data indicate that both normal and burned skin extracts evoked an antibody response when injected into rabbits and that both the normal and the burned tissue extracts appeared to contain closely related antigens as defined by the Ouchterlony technique, some of which could be demonstrated in the blood of burned mice.

The concept that there is no apparent difference in the antigens present in burned tissue compared to normal tissue is of importance. Investigators have been trying to isolate a specific antigenic burn toxin for years. The

isolation of such a toxin was thought to be important as a rationale for the use of convalescent serum from burned patients as a therapeutic agent[1,2]. Our data do not support the thought that there is a specific antigenic toxin unique to burned tissue. It is suggested that tissue antigens of normal skin may play a role in the acute mortality observed after thermal injury. Acute mortality may be, in part, due to the fact that some normal tissue components which are usually intracellular are released into the blood upon burn injury where they act as antigens and in addition, exert a deleterious effect. The nature of these tissue components and the mechanism of their action is unknown at the present time, however antibodies formed against these components may neutralize harmful effects of these antigens and thereby reduce mortality following burns. Studies of these antigens and their toxicity are in progress.

Zusammenfassung. In Extrakten normaler und verbrannter Haut von Mäusen wurde kein Unterschied zwischen den Antigenen gefunden. Kurze Zeit nach der Verbrennung wurden Antigene auch im Serum festgestellt.

I. A. Holder and M. Jogan

Departments of Microbiology and Surgery, University of Cincinnati College of Medicine and Shriners Burns Institute, 202 Goodman Street, Cincinnati (Ohio 45219, USA), 6 July 1970.

Antigenic Changes During the Life Cycle of *Plasmodium falciparum*

Immunofluorescence (IF) studies have shown that the asexual forms of *P. falciparum* contain a cytoplasmic antigen which reacts with an antibody that is commonly present in the sera of patients who have had repeated infections with *P. falciparum* malaria[1-3]: a similar antigen is present as 'stippling' in the cytoplasm of erythrocytes infected with asexual parasites[1,4]. We now present evidence that this antigen is not detectable in the cytoplasm of immature and mature gametocytes of *P. falciparum* and thus demonstrate that there is marked antigenic difference between parasites in the asexual and sexual cycles.

The organisms were studied in the asexual cycle a) as small ring forms and intermediate trophozoites in the peripheral blood of 6 patients with acute attacks of malaria and b) as late trophozoites and schizonts, either naturally occurring in the blood of 3 infected placentas[5] or differentiated during culture in vitro[6] of peripheral blood removed from 4 patients during an acute attack of malaria. Since immature gametocytes of *P. falciparum* are only rarely found in the peripheral blood, the sexual cycle was studied in the abundant viable immature and mature gametocytes in the haemorrhagic ascitic fluid of a Gambian patient with carcinoma of the liver[7]: the findings were confirmed by study of peripheral blood containing scanty mature gametocytes from 3 children recovering from acute attacks of malaria.

The organisms were stained with the indirect IF method[8] in unfixed thin blood films with the sera of 6 Gambian adults hyperimmune to *P. falciparum* followed by fluorescein-conjugated goat anti-human-γ-globulin serum (Microbiological Associates Inc.); the sera of healthy Europeans, who had never had malaria, were used as controls. Preparations were examined with dark-ground illumination by invisible ultraviolet light either alone or in combination with visible orange light: under the latter conditions, attached dye fluoresced green and malaria pigment appeared orange. Certain stages, such as ring-form trophozoites, schizonts and mature gametocytes, were easily identified under ultraviolet illumination, but others, such as large trophozoites and immature gametocytes were distinguished by the distribution of malaria pigment (Figure 1), which was clumped in the former and dispersed in the latter[9].

At all stages of the asexual cycle, the organisms showed specific IF staining of the cytoplasm but not of the nuclei, pigment or vacuoles; there was also linear or particulate IF staining (similar to Maurer's clefts in size and distribution) in the cytoplasm of erythrocytes infected with organisms in the asexual cycle (Figure 2).

At all recognizable stages of the sexual cycle, the parasites were completely unstained. However, in the cytoplasm of erythrocytes infected with gametocytes there was widespread intense IF staining: this was confluent in the early immature gametocytes, but with late immature or mature gametocytes there was an unstained cleft, usually on the concave side of the parasite (Figure 3). It is probable that we were detecting the same antigen in the different situations, since each of 6 immune sera showed IF staining of all positive components to the same titre, irrespective of the age or stage of the *P. falciparum* parasites used as antigen. The non-immune sera never gave IF staining of erythrocytes or *P. falciparum* parasites.

Our IF observations on the antigenicity of *P. falciparum* confirm the previous reports on the asexual cycle[1,2,8], but contradict the only report on the sexual cycle, namely

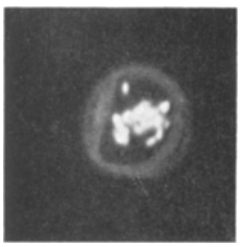

Fig. 1. Immature gametocyte of *P. falciparum* identified by the distribution of pigment using dark-ground double illumination. The orange light reflected by the pigment appears white and the fluorescent green light appears grey. × 2000.

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