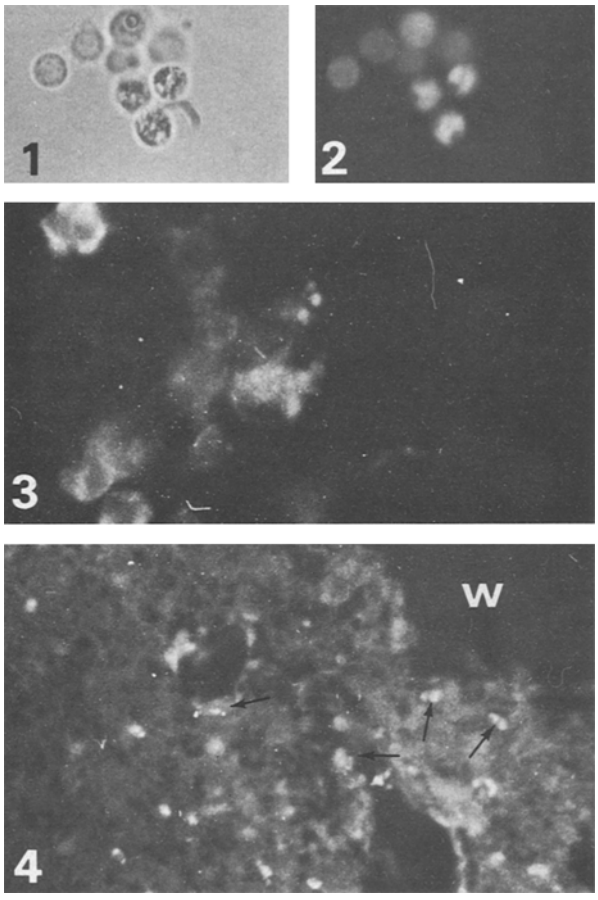

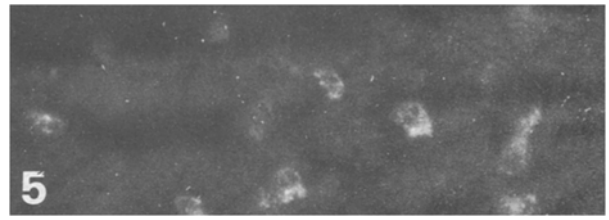

Fig. 1. Rat peritoneal macrophages 5 days after i.p. injection of paraffin. After formaldehyde fixation the cells were incubated with rabbit antiserum against rat peritoneal macrophages, followed by incubation in TRITC-labeled goat antiserum against rabbit IgG. Then, the cells were incubated with naphthyl-AS-BI-phosphate and Fast Red TR salt to demonstrate nonspecific esterases. There is an intense activity of nonspecific esterases in the cytoplasm of most peritoneal macrophages. Light microscopy, unstained. ×580. Fig. 2. Rat peritoneal macrophages as shown in figure 1. Fluorescence microscopy to demonstrate TRITC-fluorescence. There is a moderate cytoplasmic fluorescence, indicating the presence of antigens reactive with the anti-macrophage antiserum. There is a close correlation between the localisation of nonspecific esterases, as shown in figure 1, and the anti-macrophage fluorescence in figure 2, indicating a true cytoplasmic distribution of the antigen. ×580. Fig. 3. Rat thymus incubated to show macrophage-specific antigens. There is an intense cytoplasmic fluorescence of TRITC in several macrophages. The surrounding thymocytes display no specific fluorescence. Fluorescence microscopy. ×950. Fig. 4. Cerebral stab wound 3 days after injury, incubated with anti-macrophage antiserum. Several small cells in the immediate surrounding of the stab wound (W) display an intense cytoplasmic fluorescence of TRITC (arrow). Fluorescence microscopy. ×230. Fig. 5. Fluorescent macrophages surrounding a cerebral stab wound in a rat 3 days after injury. The fluorescence is cytoplasmic and there is no fluorescence in neurons or neuroglial cells. Fluorescence microscopy. ×580.

differentiation into blood monocytes, histiocytes, etc, results in a variable ultrastructural appearance. Studies are in progress to characterize further the antigens and the antiserum, including localisation at the cellular and subcellular level.

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HBsAg uptake by macrophages in vitro: An immunofluorescence study[1]

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Summary. The uptake of HBsAg by in vitro cultured macrophages was studied by immunofluorescence method. Intracytoplasmic fluorescent particles appeared 3 h after the contact with HBsAg-positive serum, while after 24–48 h only a few cells contained these particles, which are probably destroyed within the cytoplasm.

Australia antigen (HBsAg) is a particle strictly associated with serum hepatitis[3], which does not contain nucleic acids, so that it has been considered as a defective virus or an antigenic determinant of a virus able to cause hepatitis[5,6]. It is well-known that the presence of HBsAg in the serum is directly related to its localization in the liver cells, while other tissues, such as bone marrow, kidney and cells obtained from bile and duodenal drainage, do not contain

it[6]. On the other hand, the exact intracellular localization of HBsAg remains in question. In fact, by the direct and indirect immunofluorescence methods, the antigen was found by some authors within the nucleus and on the nuclear membrane[6-8], while it was localized in the cytoplasm by others[9].

Moreover, we do not possess any finding concerning the fate of the HBsAg within the cell. As to the presence of HBsAg in the serum, it may be particularly interesting to know if the macrophage system constitues a protective host system, or if it is a model of virus spreading, as has been observed with other viruses[11]. Then, it seemed of interest to study the behaviour of HBsAg after its uptake by human and mouse macrophages, in order to elucidate the mechanism of its penetration into the cell and its intracellular localization under more controlled biological conditions by using the direct immunofluorescence technique. The experiments were carried out under conditions as similar as possible, so that we could compare the behaviour of HBsAg in cultures of cells derived from different sources.

Materials and methods. Animals. 8-week-old male albino mice (Swiss strain), weighing about 40 g, were used.

Cell cultures. Peritoneal mouse macrophages: A macrophage cell suspension was obtained according to the technique of Mallucci[12]. Monolayer cell cultures were grown in Leighton tubes containing a cover slip, for 7 days in a 37 °C incubator. Renewal of medium was made every 2 days. The nutrient medium consisted of 75% Parker's 199, supplemented with 15% fetal calf serum (Microbiological Associates) and 10% lactalbumin hydrolisate (NBC). Human macrophages: Cultures of human macrophages were obtained following the method of Chang and Andersen[13].

Reagents. Fluorescein-isothiocyanate conjugated rabbit anti-HBsAg serum (Behringwerke) was used for the direct fluorescent staining of cell cultures.

Source of HBsAg. Sera from patients with HBsAg-positive acute or chronic hepatitis and from apparently healthy HBsAg carriers were used. The sera were diluted, for the experiments, at 10% concentration in the respective medium.

Experiments. The nutrient medium was eliminated from the tubes and replaced with the HBsAg-positive serum dilution to be tested. After 30 sec, 1 h, 3 h, 6 h, 24 h and 48 h this solution was replaced with the specific labelled antiserum for 30 sec and then the cell cultures were washed and fixed in a glutharaldehyde 2.5% solution for 30 sec at +4 °C. Controls for each experiment were carried out by this way: a batch of cells was treated by replacing the nutrient medium with plain fluorescent rabbit antiserum, while another batch of cells was treated first with unconjugated antiserum and then with fluorescein-conjugated HBsAg antibodies (blocking test).

The specimens were then observed under a Leitz Orthoplan microscope equipped with a device for fluorescence in incident light following Ploem by using a BG 12 exciter filter and a barrier filter. Photographs were taken on a Kodak Ektachrome 200 color film.

Results. In our experiments, we observed no differences between the behaviour of mouse and that of human macrophages in the phagocytizing activity of HBsAg particles. After 30 sec and 1 h of contact with HBsAg-positive serum, we could detect an apple-green fluorescence on the surface of the cell membrane, while the cytoplasm showed only a pale, diffuse nonspecific fluorescence. After 3 h and 6 h of incubation, bright-yellow fluorescent particles appeared inside the cells, mainly scattered within the cytoplasm (figure 1). These fluorescent granules varied considerably in size and number. It must be noticed that about 70% or more of the human cells showed inside their cytoplasm 2 or more fluorescent granules. Considering that

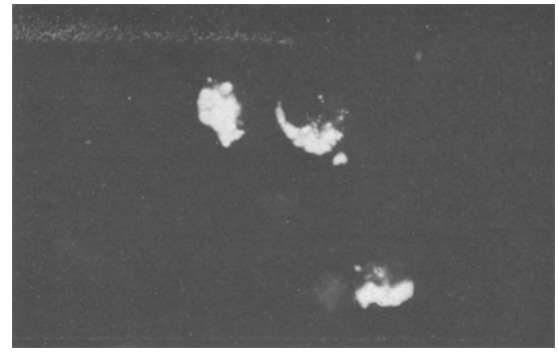

Fig. 1. Human macrophages after 3 h of incubation with HBsAg-positive serum.

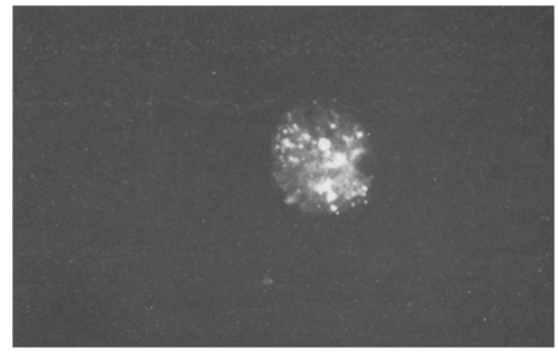

Fig. 2. Human macrophage after 24 h of incubation with HBsAg-positive serum.

human macrophage cultures are obtained from a nonhomogenous population, we believe that this percentage is in relation to the number of macrophages in the cultures. When the incubation with HBsAg was prolonged for 24 h and 48 h, only a few cells contained intracytoplasmic particles exibiting a weak orange-yellow fluorescence (figure 2). Control cells did not show any intracellular fluorescent particle. HBsAg was never detected inside the nucleus or on the nuclear membrane of the macrophagic cells. A light-green diffuse cytoplasmic fluorescence was sometimes observed both in the test and in the control slides, but it was referred to a nonspecific staining.

Discussion. Macrophages play an important role in the host defence against bacterial agents, and it seems that they are essential in generalized viral infections too. Blanden[14] showed that macrophages are a necessary component required for full expression of the viral suppressive effect of immune lymphocytes. In particular, the function of macrophages in the mechanisms of immune response is represented by the uptake and digestion of bacteria.

In our experiments, HBsAg particles were phagocytized by mouse and human macrophages. This finding could support the hypothesis that macrophages are able to engulf viral particles too, and it is in accordance with the findings of other authors which demonstrated that liver macrophages take up ectromelia virus within a few min after its i.v. injection in mice[11]. In the same way, it was demonstrated that P^{32}-labelled vescicular stomatitis and Newcastle disease viruses, injected in mice, rapidly disappeared from the blood while they were concentrated in the Kupffer cells of the liver[15]. Depending on the nature of the virus and on the species of animal host, macrophagic cells can either destroy the viral particles or support their intracellular

multiplication. For example, the virulence of some virus strains, e.g. ectromelia virus[16] and encephalomyocarditis virus[17], is associated with the capacity of growing in mouse macrophages. It has also been demonstrated that, in the mouse[11], ectromelia virus is rapidly removed from circulation by Kupffer cells, multiplies in them and then spreads to the parenchymal cells of the liver: growth in liver macrophages seems to be an essential preliminary to the hepatic cell infection. On the other hand, this same author demonstrated that different viruses, such as vaccinia, influenza and mixoma viruses in the mouse, and ectromelia virus in the rat, are taken up by the liver macrophages and then they fail to reappear. This was interpreted as an intracellular destruction of virus particles within the digestive vacuoles of the macrophage cell.

In conclusion, it seems that, during viral infections, macrophages can act either as vehicles of viruses or as viricidal cells. Therefore, it may be supposed that, for a certain species, most nonpathogenic viruses are taken up and destroyed by macrophages, while pathogenic viruses are able to multiply in these cells. Since in our experiments HBsAg particles were no more detectable within phagocytizing cells after long time incubations (24 and 48 h), it is conceivable that macrophages destroy the antigen respectively in the mouse and in the man. However we are unable to exclude the possibility that antigen particles could be present within the cells under a masked or defective form which is not detectable by the immunofluorescence method. Further studies are being carried out to elucidate this problem.

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Carcinogenicity examination of betel nuts and piper betel leaves[1]

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Summary. A dry powder of betel nuts, piper betel leaves and lime was administered to rats. Epidermal thickening was frequently observed in the upper digestive tracts of rats in groups fed the betel nut diet mixed with lime and the betel leaves diet, and a forestomach papilloma was seen in 1 rat given betel leaves diet. These epidermal changes were scarcely seen in rats given either betel nut or normal diet alone.

A high incidence of oral cancer is recorded in South-East Asian countries. Many investigators[2-7] suggested that the high incidence of oral cancer is due to the habit of chewing betel quid containing betel nut, betel leaf, lime and other organic materials. Attempts to confirm the carcinogenic activities of the betel quid using experimental animals have also been made. It was reported that mice in which extracts of betel quid with tobacco were painted in the ears[8] or instilled in the vaginae[9], developed a low incidence of papilloma and squamous cell carcinoma at the treated sites. Similar changes were observed in the hamster buccal pouch[10] treated with extracts of betel nut alone or in combination with tobacco. However, another investigator[11] could not induce tumours in the hamster buccal pouch treated with the pellets that contained betel quid ingredients. Recently, Ranadive et al.[12] and Kapadia et al.[13] reported that s.c. malignant tumours were induced in animals by s.c. injection of aqueous extraction from betel nut. Thus, carcinogenicity examinations of betel quid have been carried out entirely by local administration of the extracted substances in animals and the results obtained were affirmative in some cases and not in others. In the present study, we tried to administer a dry powder of betel nuts, piper betel leaves and lime, which are the main ingredients of betel quid, separately or in combination, to rats by feeding and examined the histopathological changes of the organs including the upper digestive tracts to know what constituents might play an important part in the carcinogenic activity of the betel quid.

Materials and methods. The inbred strain ACI rats of both sexes, 1.5 months old, were divided into 4 groups and treated as follows. Group I. 8 male and 8 female rats received betel nut diet until termination of the experiment (480 days). To prepare the betel nut diets, betel nuts imported from Indonesia was dried, milled and mixed with a rat basal diet CE-2 (CLEA Japan Inc., Tokyo) in 20% of the total. Group II. For 480 days, 11 male and 8 female rats received the diet containing 20% of dry powder of betel nut and 1% of calcium hydroxide. Group III. 9 male and 8 female rats were fed a diet containing 20% of dry powder of piper betel leaves imported from Formosa, for 300–327 days. After the termination of feeding of the experimental diet, rats were returned to the normal diet. Another group of 9 males and 10 females served as controls; they were fed the normal diet. All the animals were autopsied at death or