Further Evidence Concerning Macrophages Producing 19 S-Antibody in Mice

In a previous communication we described macrophages which produced in mice 19 S-Antibody against SRBC[1]. This result was obtained by a modification of the plaque technique of JERNE and NORDIN[2] and a morphological study of the plaque-forming cells[1]. In further experiments we combined cytological observations with functional studies on these plaque-forming cells.

The morphological aspect of the plaque-forming cells indicates that some of these cells are macrophages. If it is so, then these cells should demonstrate phagocytic activity. The purpose of this study is therefore to show that plaque-forming cells engulf erythrocytes and that they can phagocytize particles while producing antibodies.

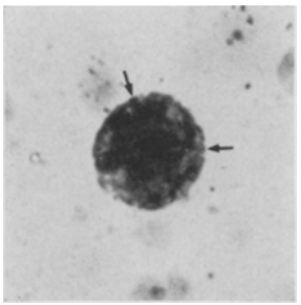

Fig. 1. Plaque-forming cell with phagocytized ink particles in the cytoplasm (small dark points, 2 indicated by arrows. Pappenheim-staining. × 1850.

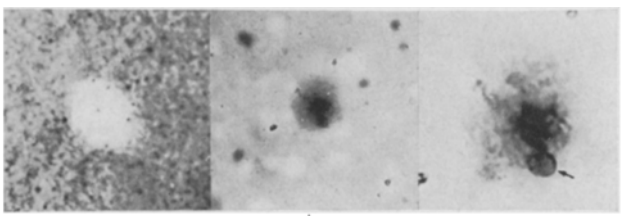

Fig. 2. Plaque-forming cell, 3 days after immunization: phagocytized erythrocyte and ink particles in the cytoplasm (arrow). Pappenheim-staining. a, × 180; b, × 950; c, × 1850.

NMRI mice, random bred, females weight 25 g were immunized i.v. against 10^8 SRBC. The mice were killed 3 and 4 days after immunization and the plaque-forming cells prepared[1,2]. 12 h before sacrifice the mice were i.v. injected with 0.2 ml of a 25% solution of Indian ink in saline (Günther Wagner, Hannover, West-Germany, 21 A 896, particle size 20–60 nm). In another experiment the prepared spleen cells of immunized mice were incubated in vitro with 1 ml of a 1% solution of Indian ink for 30 min at room temperature. After the incubation time the plaque assay was performed. The number of plaque-forming cells was not influenced by the exposure to the Indian ink. The cells were stained by the Pappenheim-method.

Results. (1) Plaque-forming cells had phagocytized ink particles in vitro. The uptake was observed mostly in cells with a large nucleus with most basophilic cytoplasm (Figure 1). (2) Plaque-forming cells exposed to ink particles in vivo incorporated these particles in their cytoplasm. (3) Furthermore we observed that plaque-forming cells phagocytize not only ink particles but also erythrocytes (Figure 2).

These observations show that antibody producing cells in the early immune phase can perform phagocytosis during the antibody production[3].

Zusammenfassung. 19-S-Antikörper-bildende Zellen können während der Antikörperbildung in der Frühphase einer Immunantwort wie Makrophagen phagozytieren.

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IgG as the Main Immobilization Factor in Rabbit Antiserum Against *Entamoeba*

That heat-inactivated hyperimmune rabbit sera were capable of immobilizing *Entamoeba histolytica*, was initially demonstrated by COLE et al.[1] Tests with unheated sera were oberved to cause lysis of the amoebae[2], an observation probably indicating that complement plays a role in the lytic activity of various microorganisms[3,4]. Unlike immobilization of bacteria[5], the immobilization of the *Entamoeba* sp. is reversible[6]. BIAGI et al.[7], using the immobilization reaction (IMR), demonstrated that the immobilization factor produced by the host after contact with the amoeba was the γ-globulin fraction. This paper presents evidence which show that, in hyperimmune rabbit sera, IgG is largely responsible for the immobilizing activity in 3 species of *Entamoeba* tested.

Material and methods. E. histolytica, E. moshkovskii and *E. invadens* were cultivated in a monophasic medium according to JONES[8], the first being incubated at 37 °C and subcultured every third day, while the latter 2 species were incubated at room temperature (26 °C) and subcultured every seventh day. The amoebae were harvested by centrifugation at 2500 rpm for 3 min and then washed 3 times with normal saline to rid them of most bacteria before inoculation into healthy adult rabbits. About 3,000,000 amoebae were injected on each occasion. The injections – twice s.c., followed by 3 times i.v., with a final booster dose of amoebae in Freund's complete adjuvant – were spaced at 5-day intervals. The animals were bled by the marginal ear veins 10 days after the

last injection. The sera were separated and stored at −20 °C. Blood was also withdrawn from the rabbits before immunization to obtain control sera.

Prior to testing for immobilizing activity and column chromatography, all the antisera were heated for 30 min in the water bath at 56 °C. The various sera were diluted in normal saline to determine the immobilizing titre. The IMR was carried out by incubating 0.1 ml of the inactivated antisera with an equal volume of concentrated suspension of amoebae in culture medium containing at least 600,000 amoebae/ml in test tubes. Incubation was carried out for 30 min at 37 °C for *E. histolytica* and at 26 °C for the other 2 species. For *E. histolytica*, it is essential that a young culture containing actively motile trophozoites and minimal rice starch be used. The mobility of 100 amoebae was counted and the percentage of immobilization calculated. Titres of control and hyperimmune sera were determined by the doubling dilution method.

To isolate the immobilization factor, the antisera were fractionated on columns containing DEAE-cellulose and Sephadex G-200. The eluant from each protein peak was pooled and concentrated by ultrafiltration to the original volume of serum applied to the columns. The concentrates were dialysed against normal saline. The immobilizing activities of the various peaks were tested and the identity of the protein constituents in the active fractions determined by immunoelectrophoresis using goat anti-rabbit whole serum and goat anti-rabbit IgG (Microbiological Associates, Inc.; Washington, D.C.).

Results. Titres of 1:5 were observed in the hyperimmune sera of the 3 species tested. Negligible immobilizing activities were observed in the control sera of *E. invadens*, *E. moshkovskii* and *E. histolytica* (8, 4 and 14% respectively) 3 peaks were obtained by fractionation of the normal and hyperimmune sera using DEAE-cellulose. In Table I, only peak I showed immobilizing activity in all the antisera tested. This peak contained

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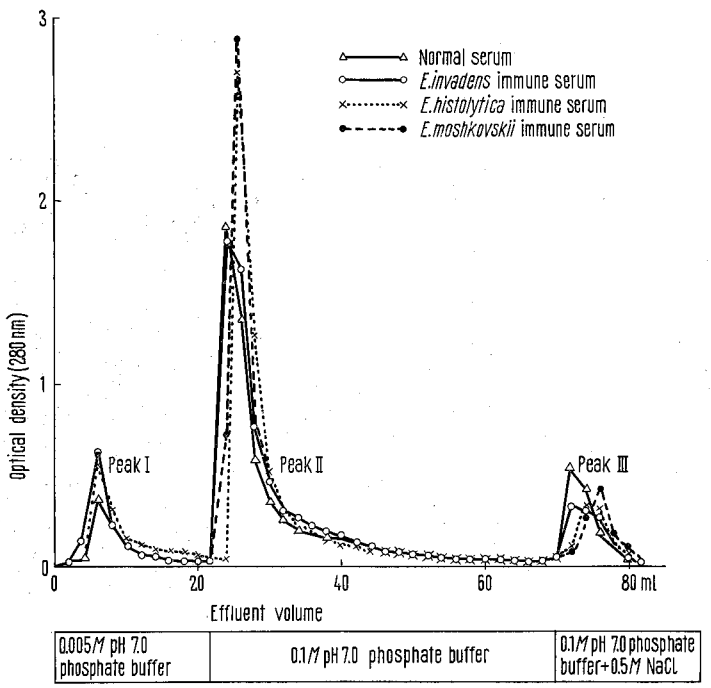

Fig. 1. Fractionation of hyperimmune and normal sera using DEAE-cellulose. 1 ml of sera was applied to the DEAE-cellulose column (1 × 10 cm) equilibrated with 0.005 *M* phosphate buffer at pH 7.0. Stepwise elution, at constant pH, was performed with increasing molarities of phosphate buffer and NaCl. 2 ml fractions were collected and the protein content read at 280 nm.

Table I. Percentage of amoebae immobilized by fractions of their respective antisera

	Fractionation of hyperimmune sera using DEAE-cellulose									Fractionation of hyperimmune sera using Sephadex G-200								
	Peak I			Peak II			Peak III			Peak I			Peak II			Peak III		
Species of amoebae[a]	EH	EM	EI	EH	EM	EI	EH	EM	EI	EH	EM	EI	EH	EM	EI	EH	EM	EI
Percentage of amoebae immobilized (%)	88	98	100	18	10	4	18	6	0	30	24	28	80	94	100	20	8	2

[a] EH, *E. histolytica*; EM, *E. moshkovskii*; EI, *E. invadens*.

IgG as demonstrated by the immunoelectrophoretic analyses using monospecific goat anti-rabbit IgG (Figure 3).

Fractionation of the antisera by Sephadex G-200, equilibrated with M/15 phosphate-buffered saline at pH 7.2, gave the usual 3 peaks[9] (Figure 2). Peak II showed high immobilizing activity while peak I displayed a low, but significant activity. Figure 4 shows IgG present in peak II and only trace amounts of IgG in peak I.

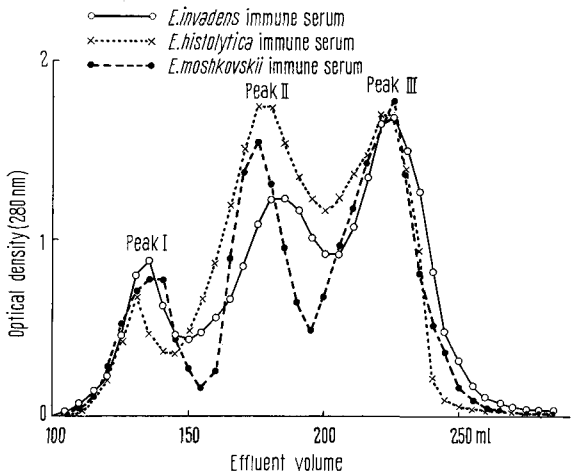

Fig. 2. Fractionation of hyperimmune sera using Sephadex G-200. 2 ml of immune sera were applied to a 2.6 × 55 cm column of Sephadex G-200 equilibrated with M/15 phosphate-buffered saline at pH 7.2. The flow rate was 30–50 ml/h.

Immobilization tests also produced a certain degree of agglutination. The immobilized cells became spherical and clumped together. This was observed mainly in the hyperimmune sera but to a lesser degree in normal serum.

In immobilization tests using the active fractions of the antisera, *E. moshkovskii* show the least cross reaction of the 3 species tested (Table II). *E. histolytica* and *E. invadens*, however, reacted mildly with their heterologous antiserum.

Discussion. Our results indicate that the immobilization factor in hyperimmune rabbit antisera is principally IgG. This does not exclude the probability that some activity, though evidently with considerably lower efficiency, is associated with the other immunoglobulin classes as well. Serodiagnostic tests, employing immunodiffusion, passive hemagglutination, and indirect fluorescent antibody tests[10,11], for *E. histolytica* in patients suffering from amoebiasis, were shown to be largely confined to the IgG fraction of their sera. It would appear that IgG is the most reactive immunoglobulin in the serology of amoebiasis in humans as well as rabbits so far studied.

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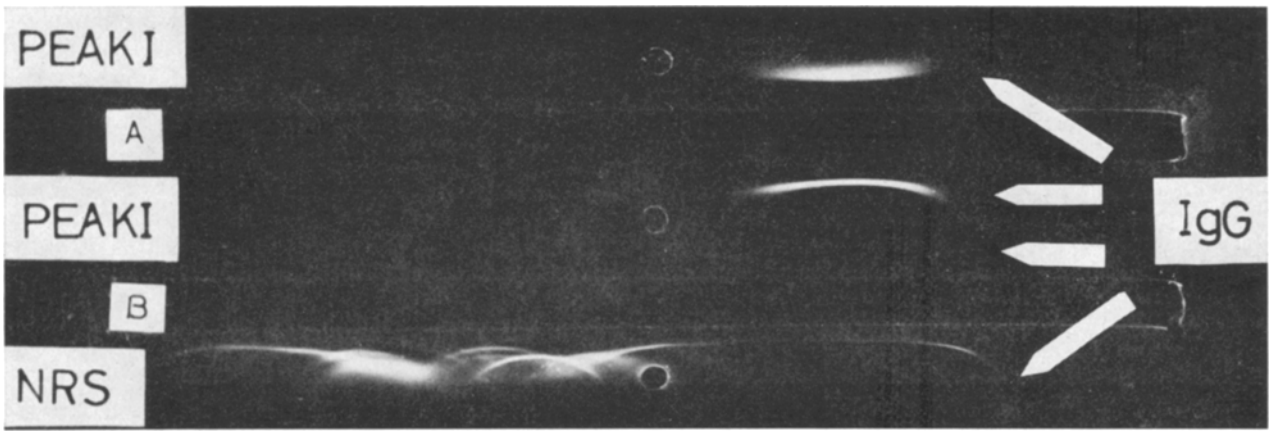

Fig. 3. Immunoelectrophoretic analyses of normal rabbit serum (NRS) and peak I obtained with DEAE-cellulose. A, goat anti-rabbit IgG; B, goat anti-rabbit whole serum.

Table II. Percentage of amoebae immobilized in cross-reaction tests between the 3 species of *Entamoeba*, using pooled, concentrated fractions of antisera containing high immobilizing activity

Antigen	Antisera					
	E. histolytica		*E. moshkovskii*		*E. invadens*	
	DEAE-cellulose Peak I	Sephadex G-200 Peak II	DEAE-cellulose Peak I	Sephadex G-200 Peak II	DEAE-cellulose Peak I	Sephadex G-200 Peak II
E. histolytica	80	76	10	16	26	22
E. moshkovskii	2	4	98	96	0	2
E. invadens	18	14	4	0	100	100

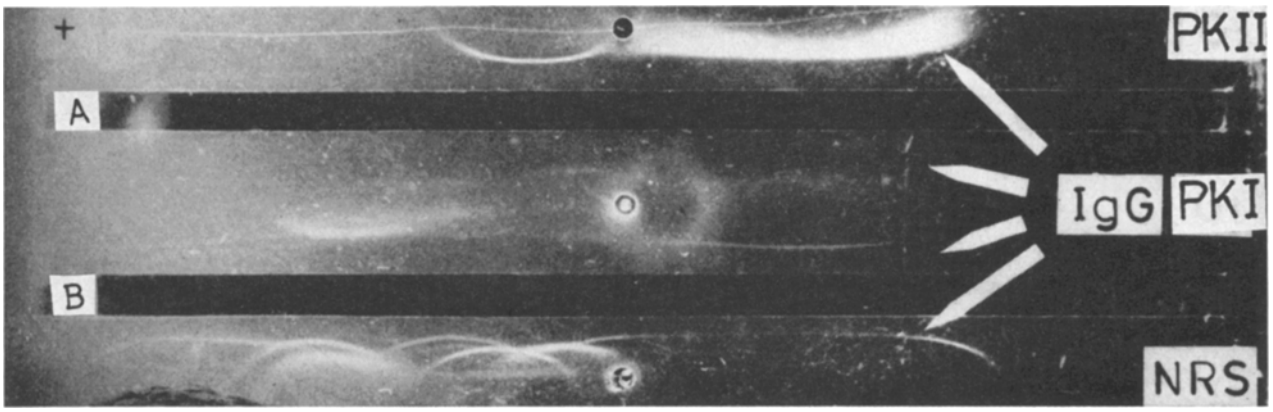

Fig. 4. Immunoelectrophoretic analyses of normal (NRS) rabbit serum and peaks I (PK I) and II (PK II) obtained with Sephadex G-200 column. A, goat anti-rabbit IgG; B, goat anti-rabbit whole serum.

A small degree of cross-reaction was observed between *E. histolytica* and *E. invadens*, an observation in agreement with that of ZAMAN[6]. *E. moshkovskii*, the only free-living species tested, show no antigenic similarity to the other 2 species.

Résumé. Les antisérums de lapin utilisées contre les trophozoïtes de l'*Entamboeba histolytica, E. moshkovskii* et *E. invadens* furent fractionnés par la cellulose DEAE et le Sephadex G-200. Les essais d'immobilisation avec ces antisérums fractionnés montrent que la fraction IgG est la plus active à cet effet. En usant les fractions actives, des essais démontrent l'existence d'une réaction croisée entre *E. invadens* et *E. histolytica.*

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Interaction Between Peripheral Blood Leucocytes and X-Irradiated Cells from Lymphoid Cell Lines

Peripheral lymphocytes from unrelated individuals when mixed in vitro are activated to blast cells which go on to synthesise DNA and enter mitosis[1]. Similar reactions occur in homologous mixtures of animal lymphoid cells[2]. The immunological significance of the reaction and its potentialities as a tissue typing test have been intensively studied in recent years[3]. The reaction is normally a two-way reaction, i.e. each population of cells stimulates and is also stimulated. Since this is obviously an undesirable complication in the interpretation of mixed cell tests there have been a number of attempts to make the reaction 'one-way' by making one donor's cells unresponsive while retaining stimulating capacity, e.g. by freezing and thawing[3,4], treatment with drugs[5,6], or X-irradiation[5,7,8]. None of these methods is ideal. However, X-irradiation appears to be the most effective way for obtaining a one-way reaction[9]. Lymphoid cells from various patients and from some normal individuals can now be maintained in continuous culture[10]. We have found that mixtures of freshly isolated human blood lymphocytes and X-irradiated lymphoid cell line cells give unusually intense interactions.

Established cell lines used in this work were kindly donated by Professor M. A. EPSTEIN[11,12], Professor G. MOORE[10] and Dr. P. GERBER[13]. The derivation of these cell lines and results of tests for the presence of herpes-like virus (HLV) are summarised in Table I. The cells were maintained in static suspension cultures in Eagle's medium supplemented with 20% human, pig, calf or foetal calf serum. Prior to irradiation the cells were suspended in fresh Eagle's medium containing 20% human serum, at a concentration of 2×10^6/ml. Cells were exposed to 6000 r of X-rays (200 kV; 12.5 mA; Filter aluminium, 1 mm; dose rate 150 r/min) and were normally used 3 h later. Preserved lymphoma cells were treated with pyruvic aldehyde or glutaraldehyde as described previously[14]. Lymphocytes were separated from

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