The Reticuloendothelial System and Immune Phenomena

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

The Reticuloendothelial System and Immune Phenomena

Proceedings of the Ludwig Aschoff Memorial Meeting
of the Reticuloendothelial Society,
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PREFACE

This volume represents a portion of the Proceedings of the Sixth International Meeting of the Reticuloendothelial Society. There is little question that the University of Freiburg was a most appropriate choice as the site of the meeting since, in essence, the Society was founded here when Aschoff undertook his classical studies on the macrophage and discovered what may well have been the last remaining biological system. In the approximate fifty years which have elapsed since Aschoff introduced the descriptive term Reticuloendothelial System to unify those cells with the common property of phagocytosis, one finds investigative activity at the highest level in all areas of reticuloendothelial involvement. Indeed, the topics covered in the present volume would of necessity require that the common property of phagocytosis which led to the formulation of the reticuloendothelial system be modified. The common basis at the present is clearly no longer phagocytic expression, but the unifying basis of host defense. The totality of reticuloendothelial involvement in host-defense is clearly reflected by the diverse scientific backgrounds and research interests of the participants of the meeting.

Remarkable advances in appreciating the involvement of the RES in maintaining the well-being of the host against a variety of endogenous and exogenous factors have been made since volume I of the Advances in Experimental Biology and Medicine on the reticuloendothelial system was published. The ubiquitous nature of the reticuloendothelial system is now being delineated in numerous laboratories with further extensions of those cells from the classical phagocytic function to a multitude of functions. Certain of these functional expressions are considered in this volume, including the structure.and origin of macrophages, chemotaxis and opsonization, phagocytosis, intracellular killing, immunity, host defense and neoplasia.

While our knowledge of the RES has advanced significantly, there are as yet many unresolved problems of the regulation, control, and expression of the RES in homeostasis, at both the experimental and clinical level. These unresolved problems of such critical biological events not only invite, but demand exploration.

It is hoped that this volume provides an expression of the present and obvious future excitement of the reticuloendothelial system in experimental and clinical medicine to students in all disciplines that are encompassed by multipotential expression of this system.

Grateful acknowledgment is made to Mrs. Marilyn Lutenbacher for her excellent secretarial services in arranging details of the Sixth International Meeting as well as her many contributions in the preparation of this book.

Nicholas R. Di Luzio

INTRODUCTION

The Sixth International Meeting of the Reticuloendothelial Society took place at the University of Freiburg, Freiburg, Germany, July 28th to August 1st. Approximately 350 participants gathered at the assembly-hall and lecture rooms of the University to attend the various scientific sessions and to take part in symposia. The Minister of Health and Welfare of the German Federal Republic and the Minister of Education for the country Baden-Wurttemberg had taken over the patronage. The Lord Mayor of Freiburg, the Rector of the University and the Dean of the Faculty of Medicine, along with representatives of the German Society of Pathology and the Society of Immunology welcomed the participants. Further greetings arrived from the Deutsche Akademie der Naturforscher Leopoldina, from the South African Society of Pathology, from the Japan Society of the Reticuloendothelial System and from the 13th International Congress of Hematology in Munich.

When, in 1968, the committee of the Reticuloendothelial Society asked me to organize the Sixth International Meeting in Germany, they were not conscious of the fact that I held an appointment to the faculty at the University of Freiburg to which Ludwig Aschoff had belonged for 30 years. Here in Freiburg he discovered a body defense system and termed it in 1924 the reticulo-endothelial system (RES). As a result of his work, he clearly became the founder of the RES research. It appeared obvious that the first conference of the RE Society to be held in Germany should take place in Freiburg in honour of this outstanding man and should be called the 'Aschoff Memorial Meeting'.

Scientific progress is based on facts and their certainty; but it also needs imagination to be able to enter the field of the unexplored. Aschoff's way of thinking was based on the foundations laid by Virchow's cellular pathology. All the more successfully then was he able to expand the limits of his subject then valid and to lay great landmarks in the new field of functional pathology.

We honour this man rightly. In his time he was regarded as the representative of his subject. In the period since Virchow's time, he has been the outstanding figure (of pathology). "I know of no one whose work has led to such important advance in so many

fields"(Robert Muir). He had, furthermore,outstanding human qualities. His English students McNee and R. Smith said of him: 'Aschoff was a noble example of all that was best in Germany.' His capability and personality attracted students from all over the world. For this reason, I was happy that scientists from 30 different countries of the world attended the Aschoff Memorial Meeting in Freiburg.

It was particularly auspicious that two of Aschoff's students were present. Professor Akazaki brought greetings from the Japan Society of the RES which he himself founded; he was the honorary president of the meeting and gave the Aschoff Memorial Lecture. Professor Buchner, also a student of Aschoff's and his successor as professor for pathology in Freiburg, greeted the meeting on behalf of the German Society of Pathology. Aschoff left a really great scientific inheritance. The conception of RES is only one of his important achievements. Whoever is working in the field of RES and reads through Aschoff's works attentively, sometimes has the impression of being able to consider himself still today as a student of Aschoff.

As in all disciplines, great progress has been made in the field of RES since Aschoff's death. Immunology has taken a particularly big step forward and has become an important field of its own today.

Finally, the participation of European research workers at this meeting is to be regarded. In this connection, the cooperation with the European Group for the Study of Lysosomes, under the encouragement of Dr. Peirre Jacques, should be mentioned. The support of this group contributed greatly to the success of the meeting. Dr. Jacques, the representative of this group, organized the very effective symposium 'Phagocytic event and lysosomal activity'. We hope that in the future the cooperation can be continued and intensified. The impressive European activity and the successful nature of the American RE Society and the Japan Society initiated the suggestion of the formation of a European Society for RES research. Plans have been developed to initiate the formation of such a European Society.

I should like to thank all the active participants again for their contribution to the success of the meeting. I owe my particular gratitude to the co-workers and colleagues who greatly aided with the organization and realization of the meeting, particularly to Mrs. Engelken, Dr. Christa Flemming, Dr. Lemperle and Dr. Nothdurft.

Kurt B. P. Flemming
Chairman,
Sixth International Meeting of
the Reticuloendothelial Society

CONTENTS

STRUCTURE AND ORIGIN OF MACROPHAGES

CHEMOTAXIS

THE PHAGOCYTIC EVENT

MACROPHAGE INTRACELLULAR METABOLISM

R E S AND IMMUNITY

R E S IN HOST DEFENSE

R E S AND NEOPLASIA

ELECTRON-MICROSCOPIC CYTOCHEMISTRY OF THE CELL COAT OF KUPFFER CELLS IN RAT LIVER

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Animal cells have an external surface layer closely bound to the plasma membrane, called the cell coat (1) or glycocalyx (for a recent survey see ref.2). This coat is usually composed of acidic glycoproteins or mucopolysaccharides (3), and is thought to play an important role in surface interactions between cells and transport phenomena across the plasma membrane.

In rat liver sinusoids three resident cell types can be distinguished: Kupffer cells, endothelial cells, and fat-storing cells (4,5). Our interest in the morphological and functional differences between these cell types, especially with regard to endocytosis, prompted us to investigate the properties of the cell coat of Kupffer cells, since this liver macrophage is thought to be the principal agent in the clearance of foreign material from the blood stream.

MATERIAL AND METHODS

Animals

Male Wistar rats weighing approximately 200 gm were used. Prior to fixation, the animals were anaesthetized with 0.2 ml Nembutal given intraperitoneally.

Fixation

(a) Perfusion fixation of the whole liver with glutaralde-

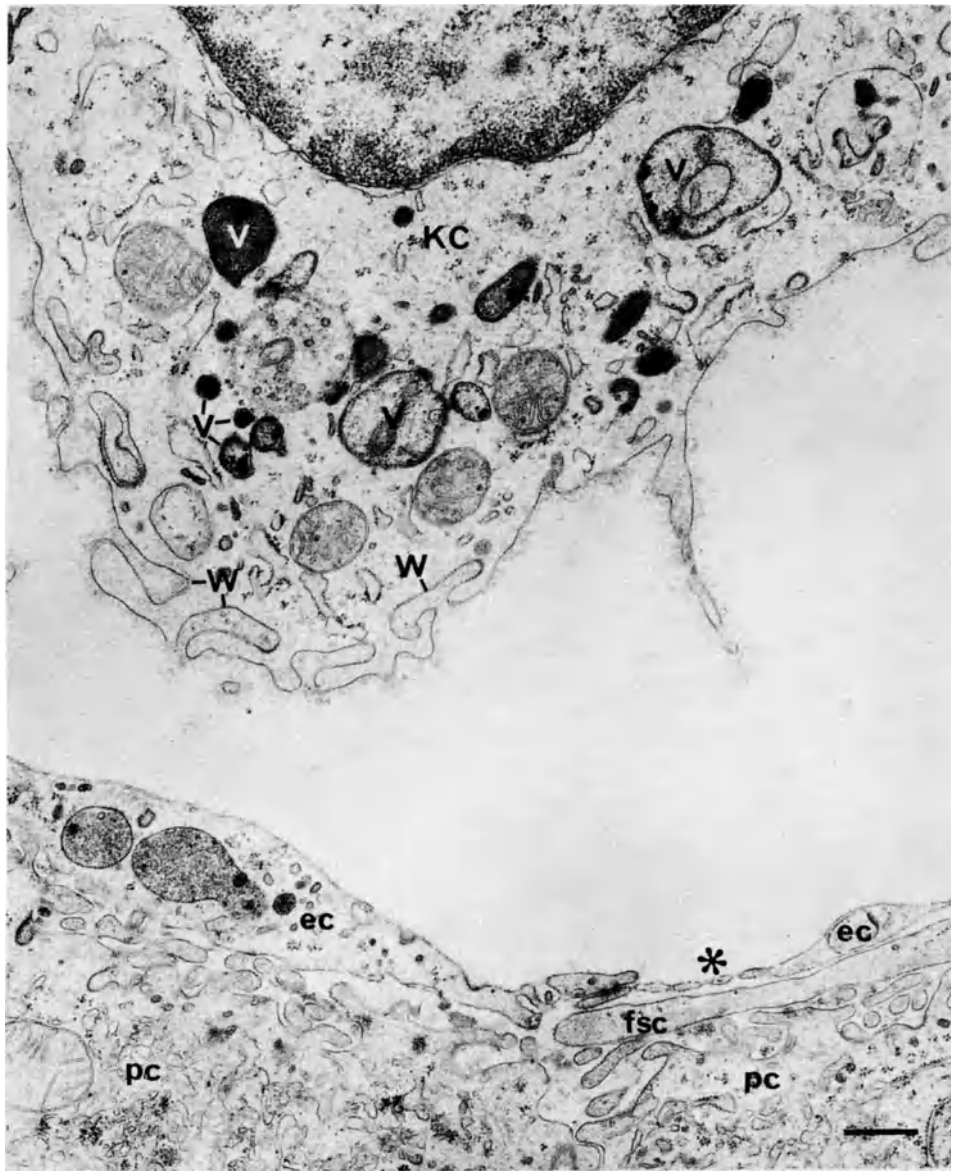


Fig. 1 Part of a rat liver sinusoid. A prominent cell coat is visible on the plasma membrane of a Kupffer cell (KC), but not on the plasma membranes of endothelial (ec), fat-storing (fsc) or hepatic parenchymal (pc) cells. Note worm-like structures (W) and numerous inclusion bodies (V) in Kupffer cell. An endothelial cell shows fenestrations (asterisk).
Frozen section, osmium immersion fixation.
Bar on this and following micrographs indicates 0.5 μ.

hyde (1.5% in cacodylate buffer, pH=7.4) for two minutes at room temperature with (a1) or without (a2) postfixation by immersion of 1 mm^3 tissue blocks in phosphate-buffered 1% osmium (pH=7.4) for one hour at 4°C. Full details of the perfusion procedure have been published elsewhere (6).

(b) Perfusion fixation of the whole liver with phosphate-buffered 1% osmium for two minutes at room temperature.

(c) Perfusion of the whole liver with buffered saline for two minutes, followed by perfusion with phosphate-buffered 1% osmium for two minutes at room temperature.

(d) Immersion fixation of 50 u frozen sections in phosphate-buffered 1% osmium for one hour at 4°C.

(e) Intravenous injection of Thorotrast (0.3 ml / 100 gm body weight); three minutes after completion of the injection the liver was fixed by method (a1). Thorotrast (Fellows Test-agar, Detroit) is a 24 to 26% suspension of positively charged colloidal thorium dioxide stabilized by dextran.

Histochemistry of acidic groups

(f) After fixation by method (a1), 1 mm^3 tissue blocks were rinsed twice in 3% acetic acid (pH=2.7; 10 minutes each), incubated for 24 hours at room temperature in a 1% (v/v) solution of Thorotrast in 3% acetic acid, and rinsed three times in 3% acetic acid (10 minutes each) and twice in cacodylate buffer (pH=7.4; 10 minutes each) (compare ref.1). To check the specificity of the staining reaction (see ref.7), tissue blocks were subjected before incubation in Thorotrast to "mild" methylation (0.8% HCl in methanol, 37°C, 3 hours) or to methylation followed by saponification (1% KOH in 80% ethanol, 37°C, 30 minutes).

(g) Whole liver was freed of blood by perfusion with buffered saline (2 minutes), fixed by perfusion with the glutaraldehyde fixative to which 1.5% alcian blue 8 GX (E. Gurr, London) had been added (8), and postfixed with 1% osmium. As controls, tissue blocks fixed by glutaraldehyde perfusion were rinsed in acetic acid (three times for 10 minutes), and then incubated for 3 hours in a 1.5% alcian blue solution at pH=2.7 (9). After rinsing in acetic acid (three times for 10 minutes) and cacodylate buffer (twice, 10 minutes each), the tissue blocks were postfixed in 1% osmium.

Care was taken to adjust the osmolality of all the solutions to approximately 320 milliosmoles.

After rapid dehydration in a graded ethanol series, the tissue was embedded in Epon. Ultrathin sections were cut on a Reichert OMU2 Ultramicrotome, stained with uranyl acetate and lead hydroxide, and photographed in a Siemens Elmiskop 1.

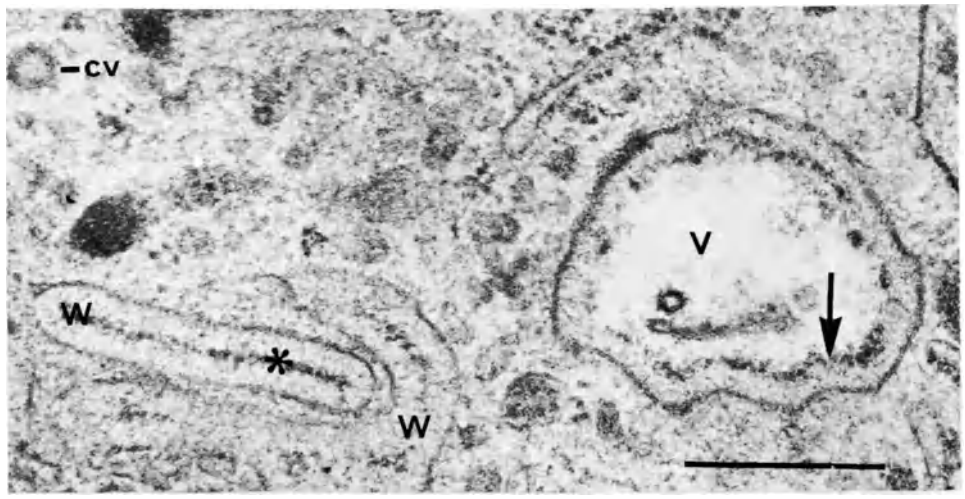
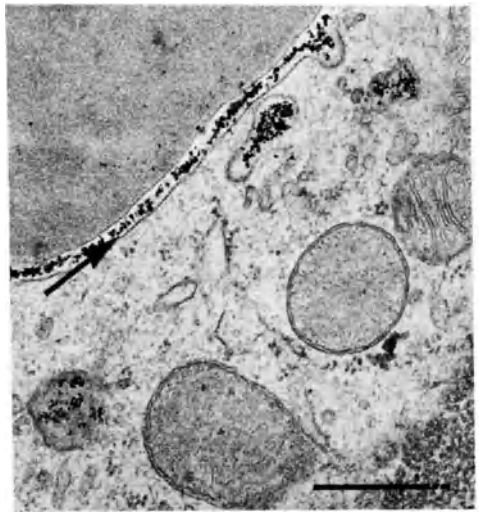


Fig.2 Part of the cytoplasm of a Kupffer cell containing worm-like structures (W) with dense midline (asterisk) and cross-striations. In an endocytic vacuole (V) the same pattern is discernible (arrow). Also present is a coated vesicle (cv). Osmium perfusion fixation.



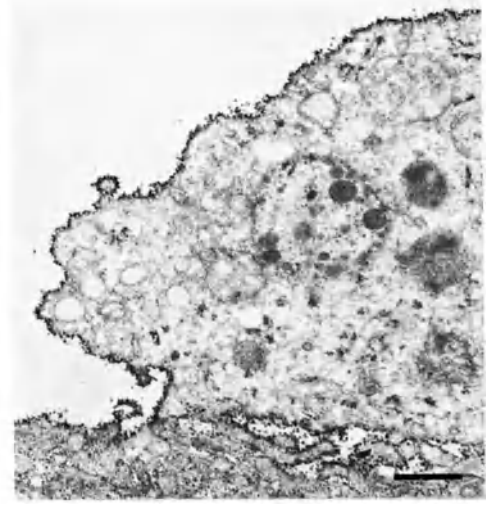


Fig. 3 (left) Part of a Kupffer cell fixed three minutes after intravenous injection of Thorotrast. The Thorotrast particles are separated from the plasma membrane by a small gap (arrow). Glutaraldehyde perfusion, osmium postfixation.
Fig. 4 Part of a sinusoid fixed before incubation with Thorotrast. The Thorotrast particles lie close to the plasma membrane, thus indicating the presence of acidic groups. Glutaraldehyde perfusion, osmium postfixation, Thorotrast incubation at pH =2.7.

Histochemistry of vicinal hydroxyl groups

Ultrathin sections of tissue fixed with glutaraldehyde only, were stained for the presence of 1,2-glycol groups by one of the following methods:

(h) silver methenamine staining (60°C, about 45 minutes) after oxidation by periodic acid (1%, 10 minutes) and chromic acid (10%, 1 minute) according to Rambourg (10) or

(i) silverproteinate staining (1%, 30 minutes) after periodic acid oxidation (1%, 10 minutes) and thiosemicarbazide incubation (1% in 10% acetic acid, 72 hours) according to Thiery (11).

For control sections, prior oxidation was omitted. The procedure according to Thiery was also performed on tissue fixed by direct osmium perfusion.

RESULTS

After glutaraldehyde fixation (a2) or glutaraldehyde followed by osmium fixation (a1), no cell coat was observed on the plasma membrane of Kupffer cells. After direct osmium fixation, however, whether by perfusion (b) or immersion (d), a distinct coat with an average thickness of 700 Å was seen over the whole surface of Kupffer cells (Fig. 1), (12). This coat was also seen when prior to direct osmium fixation, blood had been removed from the liver by perfusion with buffered saline (c). On endothelial cells, fat-storing cells, or hepatic parenchymal cells, no comparable structure was discernible.

Three minutes after intravenous injection of Thorotrast (e) the Thorotrast particles were attached exclusively to the surface of the Kupffer cells (compare ref. 13, 14). Between the attached particles and the plasma membrane, however, there was always a small gap of about 200 Å (Fig.3, see also ref.15).

After the incubation of fixed tissue blocks in Thorotrast at low pH (f), all cells showed extracellular staining of the plasma membrane. The Thorotrast particles were located very close to the outer leaflet of the unit membrane (Fig.4). No differences in staining reaction were observed between the various cell types of the sinusoidal lining. Methylation, which is thought to block carboxylic acid and to hydrolyze sulfate esters, abolished all staining reaction. After subsequent saponification, which demethylates carboxylic esters, the original staining intensity was restored.

In the first experiments with the glutaraldehyde-alcian blue mixture, prior perfusion with buffered saline was omitted

and blood therefore mixed with the fixative in the sinusoids during perfusion. This resulted in heavy, highly irregular precipitates of alcian blue-containing substance(s) on the lumenal surfaces of the sinusoidal lining cells and on erythrocytes, making any interpretation impossible. A similar phenomenon has been reported by Behnke and Zelander (16). If, however, the blood was removed by perfusion with buffered saline (g) prior to the fixing-staining procedure, no aspecific precipitation was observed (erythrocytes, for example, were no longer stained) and the staining patterns of all cells became reproducible. Under these conditions Kupffer cells had a thin (100 to 150 Å), amorphous, alcian blue-positive layer external to the plasma membrane (Fig.5). The specificity of the reaction for acidic groups of low pK was ascertained by incubating glutaraldehyde-fixed tissue blocks with a 1.5% alcian blue solution at pH=2.7: the same staining pattern resulted. The worm-like structures of Kupffer cells (ref.4,5,17-20, compare Figs.1 and 2) showed, besides staining of the 100 to 150 Å layer on their invaginated plasma membrane, increased electron density of the midline.

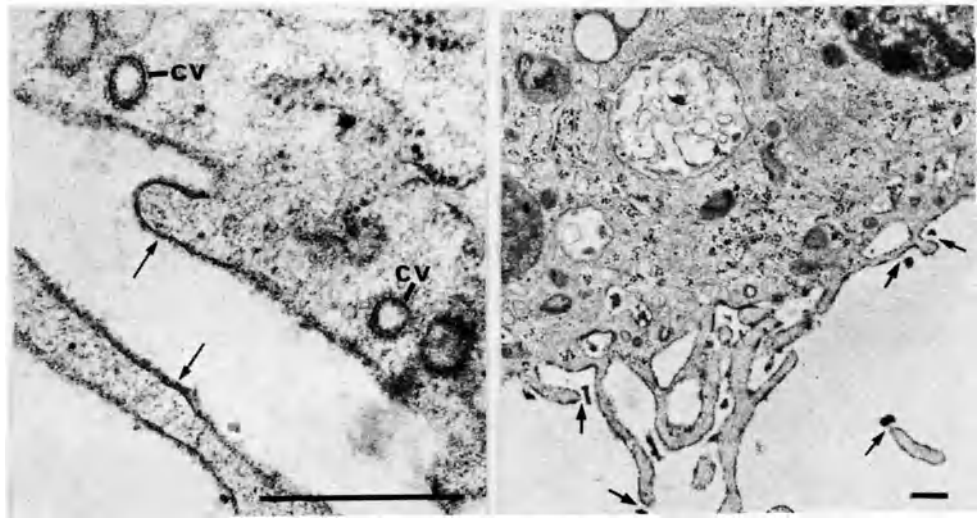


Fig.5 (left) Part of a Kupffer cell. The extracellular localization of the alcian blue-positive inner layer can be seen (arrows). Coated vesicles are present (cv). Buffered saline perfusion, followed by perfusion with glutaraldehyde plus alcian blue, osmium postfixation.

Fig.6 (right) Part of a Kupffer cell fixed by glutaraldehyde plus alcian blue without prior buffered saline perfusion. Aspecific precipitates are separated from the inner layer by an electron-lucent gap (arrows). Osmium postfixation.

Very occasionally, an irregular increase in density of the whole 700 Å surface coat of Kupffer cells was noticed.

Although, as mentioned, combined alcian blue-glutaraldehyde perfusion without prior removal of the blood gave inconsistent results, an interesting observation was made in this material: electron-dense deposits, separated by a 500 Å electron-lucent gap from the positively stained 100 to 150 Å layer, were sometimes seen on Kupffer cells (Fig.6).

Staining of glutaraldehyde-fixed thin sections with silver methenamine without prior oxidation gave (aspecific) staining of only nuclear material, ribosomes, collagenous fibrils, and an occasional vacuole. After periodic acid-chromic acid oxidation, however, many details could be seen. Among the structures stained were the plasma membranes of all cells and - in Kupffer cells - the limiting membrane, midline, and (sometimes) cross-striations of the worm-like structures (Fig.7). Cytoplasmic membranes of mitochondria, endoplasmic reticulum, nucleus and peroxisomes did not show silver deposition.

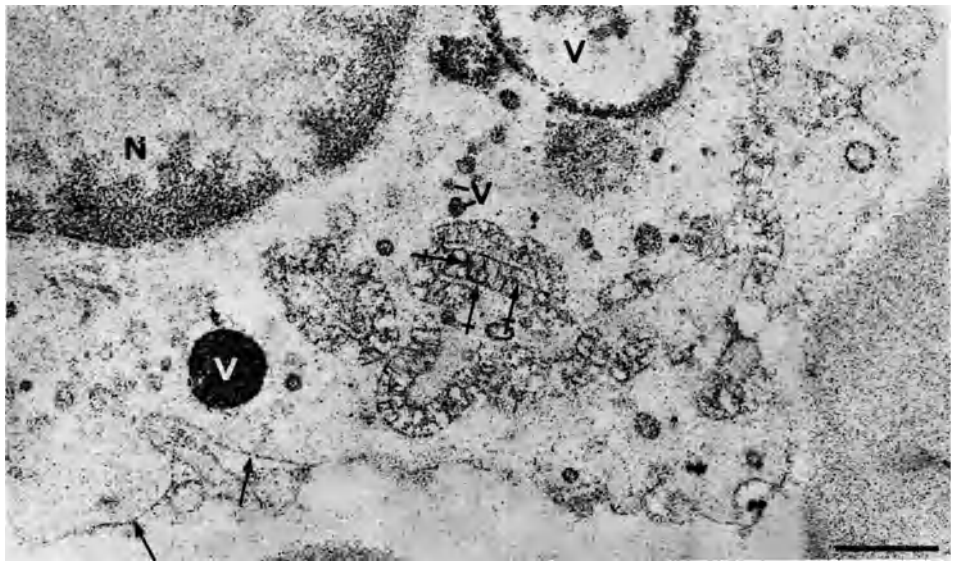
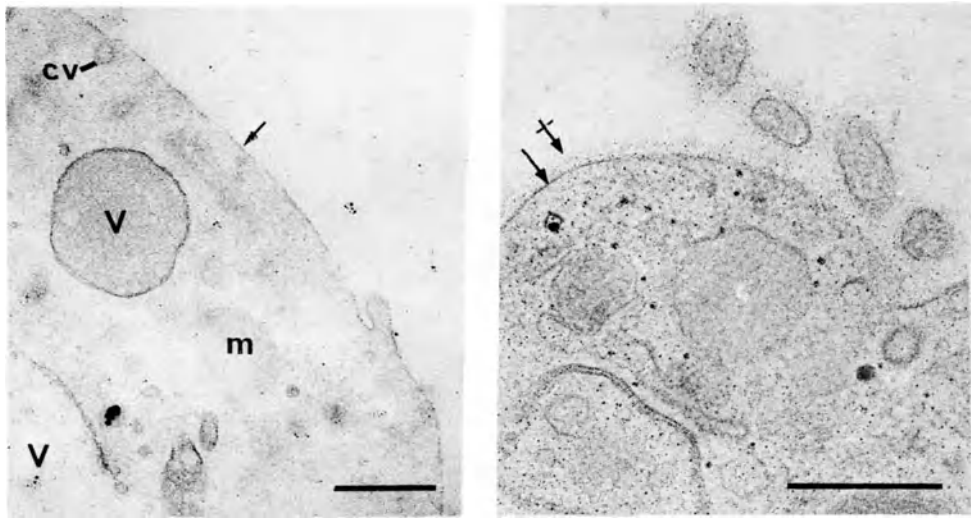


Fig.7 Part of a Kupffer cell containing worm-like structures stained with silver methenamine. Plasma membrane of the Kupffer cell (arrows) as well as the membrane, midline, and cross striations of the worm-like structures (barred arrows) react positively. Also stained are numerous inclusion bodies (V) and nuclear material (N). Glutaraldehyde perfusion; periodic acid - chromic acid - silver methenamine staining.

Thiery's silver proteinate staining (i) gave exactly the same results (Fig.8). Although the application of silver staining methods to osmium-fixed tissues is histochemically unsound (10), Thiery's method was also applied to tissues fixed by osmium perfusion, since in this material the whole 700 Å cell coat is still dimly visible even after partial removal of the osmium by periodic acid. Here too, silver deposition was observed only close to the plasma membrane and the remainder of the cell coat did not take the stain (Fig.9).

DISCUSSION

A "thick" (>200 Å) cell coat ("fuzzy coat") has been observed repeatedly on the external plasma membrane of epithelial cells, e.g. of intestine, gall bladder, urinary tract, or oral cavity; compare also the mucoid layer on amebae (references can be found in 2). On the plasma membrane of cells exposed to the "milieu interieur", however, only one instance of the presence of a fuzzy coat has been reported sofar:



Figs. 8 and 9 Parts of Kupffer cells stained by Thiery's method (periodic acid-thiosemicarbazide-silverproteinate).
Fig. 8 (left) Tissue fixed by glutaraldehyde perfusion only. Note stain on plasma membrane (arrow) and limiting membranes of inclusion bodies (V) and coated vesicle (cv). Membranes of mitochondrion (m) are unstained however.
Fig. 9 (right) Tissue fixed by osmium perfusion. The differences in staining reaction between inner (arrow) and outer (barred arrow) layer of the cell coat can be seen. The inner layer reacts positively, but the outer layer shows only the same background density as the cytoplasmic matrix.

both Röhlich and Törö (20) and Nossal et al. (21) described a filamentary layer with a thickness of 500 (20) or 200 to 1000 (21) Å on lymph node medullary macrophages. Moreover, Röhlich and Törö (20) have clearly shown the role of this cell coat in the attachment of chylomicron particles and in the formation of worm-like structures (see their fig.3).

With regard to Kupffer cells, the presence of a comparable fuzzy coat has been inferred from observations on the attachment of colloidal particles (13; see also 15) and the morphology of worm-like structures (17), and Orci et al. (18) observed fuzzy material in and around these structures. Histochemical observations on the cell coat of Kupffer cells based on the use of alcian blue plus lanthanum (22) did not disclose any difference from the coating on other hepatic cells.

We think our results confirm and extend these observations and allow the construction of the following model of the Kupffer cell coating: the fuzzy coat of Kupffer cells is a 700 Å thick structure, composed of two layers. One of these (the inner layer, thickness 100 to 150 Å) is closely apposed to the external electron-dense leaflet of the cytoplasmic unit membrane. This inner layer reacts positively with stains for acidic groups of low pK (Thorotrast, alcian blue) and for vicinal hydroxyl groups (silver methenamine, silverproteinate), and is therefore presumably composed of acidic glycoproteins or mucopolysaccharides. The other layer (outer layer, thickness 500 to 600 Å) is external to the afore-mentioned layer and does not react with the stains used. The chemical nature of this layer is still unknown. The presence of a small gap between the plasma membrane of Kupffer cells and the Thorotrast particles attached to these cells after intravenous injection supports the concept of a two-layer structure.

The invisibility of the fuzzy coat after glutaraldehyde fixation is puzzling. However, we assume that this absence is an optical illusion: the presence in tissue fixed with the glutaraldehyde-alcian blue mixture without prior buffer perfusion of a 500 Å-wide gap between the alcian blue-induced aspecific precipitates and the inner layer of the cell coat of Kupffer cells, demonstrates the continued presence of the outer layer even after glutaraldehyde fixation.

The diameter of the worm-like structures (in our material about 1400 Å) equals twice the thickness of the cell coat, thus confirming the postulated mechanism for the formation of worm-like structures (17,20). This form of endocytosis, which seems to be specific for macrophages (15, 17-20) provides these cells with a mechanism for the rapid and selective (23) uptake of material from their surroundings.

The nature of the outer layer of the cell coat (non-glycoprotein and non-mucopolysaccharide, a composition which to the best of our knowledge has not yet been reported for any other mammalian cell coat) could be crucial for the discriminatory power of macrophage endocytosis.

SUMMARY

By morphological and histochemical methods, Kupffer cells of rat liver were shown to have a 700 Å thick filamentous cell coat. This cell coat (fuzzy coat) is composed of two layers: one layer (thickness 100 to 150 Å) lies close to the plasma membrane and is composed of acidic glycoproteins or mucopolysaccharides. The other layer lies external to the afore-mentioned layer and is about 500 to 600 Å thick; its composition is still unknown.

The authors are indebted to Mr. J. van der Meulen and Mr. J.M. van 't Noordende for technical assistance, and to Dr. W.Th. Daems for critically reading the manuscript and giving helpfull suggestions.

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SPREADING OF MACROPHAGES ON GLASS AND ITS RELATION TO RECOGNITION AND THE CELL COAT

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Introduction

There is a layer of material on the outside of the cell membrane of many cells, largely composed of carbohydrate and demonstrable, sometimes by conventional E.M. techniques (Yamada 1955) and often by specialized E.M. cytochemical techniques. This layer has been called the "glycocalyx" (Bennett 1963) or cell coat (Rambourg and Leblond 1967). In the amoeba such a coat has been shown to play an important part in the first stage of phagocytosis and therefore must be involved in the recognition of foreign material (Brandt and Pappas 1960).

There are some similarities between spreading on glass and phagocytosis; in a way the phagocyte is attempting to phagocytose the glass surface. The present report describes the cell coat of the mouse peritoneal macrophage and attempts to relate the coat to spreading on glass and recognition of artificially aged autologous red cells. This work is fully reported with a fuller citation of the literature elsewhere (Carr and Carr 1970; Carr et al. 1970).

Materials and Methods

The cells used were obtained from the peritoneal cavity of outbred male white mice. After glutaraldehyde fixation they were stained with ruthenium red, embedded in Araldite, sectioned, and examined in the transmission E.M. Specimens were examined after washing six times in Hanks solution, and after trypsinization, (100 μG/ml.) in a roller tube at 37°C for 1 hour, methylation

and digestion with phospholipase, amylase, hyaluronidase, and neuraminidase.

The attachment of glutaraldehyde fixed red cells to macrophage monolayers was studied by the technique of Rabinovitch (1967). In brief macrophages were allowed to settle on coverslips; these were then incubated *in vitro* in a Petri dish with added fixed red cells at a concentration of 5000/cu mm. Macrophages were examined both with and without trypsinization; trypsinization was carried out both on cell suspensions and on settled monolayers.

The process of spreading was studied using phase contrast, and time lapse photomicrography, and by scanning E.M. after glutaraldehyde fixation and carbon/platinum or gold palladium shadowing. Both normal cells and cells from the peritoneal cavity of animals stimulated with glyceryl trioleate were examined.

Results

After ruthenium red staining there is an electron dense layer on the surface of the cell membrane of the macrophage, outlining indentations of various sizes at the cell surface. These are often crossed by fine strands of coat material. This coat is 120A long separated by areas of lesser thickness, and shows an ill defined mosaic appearance in grazing sections. Globular aggregates of coat material up to 750A in diameter lie on the surface (Figure 1).

Staining is inhibited by methylation but not by washing, trypsinization or consistently by the other enzymes used.

Aldehyde fixed red cells adhere to macrophages as shown by Rabinovitch (1967). This adherence or recognition is grossly reduced by washing or trypsinization but largely restored by incubation in mouse plasma (Table 1).

No difference in the rate of translatory movement was seen between normal and triolein stimulated macrophages once the cells were settled; however triolein stimulated cells settled on glass to the fully extended form much more rapidly than normal controls. Trypsinization did not abolish settling on glass (Figure 2).

Discussion

These results establish that the mouse peritoneal macrophage has a prominent cell coat and define its structure. It is thin - much thinner than that of intestinal epithelial cells - and highly resistant to enzymatic digestion, at concentrations which do not totally disrupt cellular architecture; it probably consists largely of acidic mucopolysaccharides. The outstanding peculiarities about it are the fine strands which cross cellular

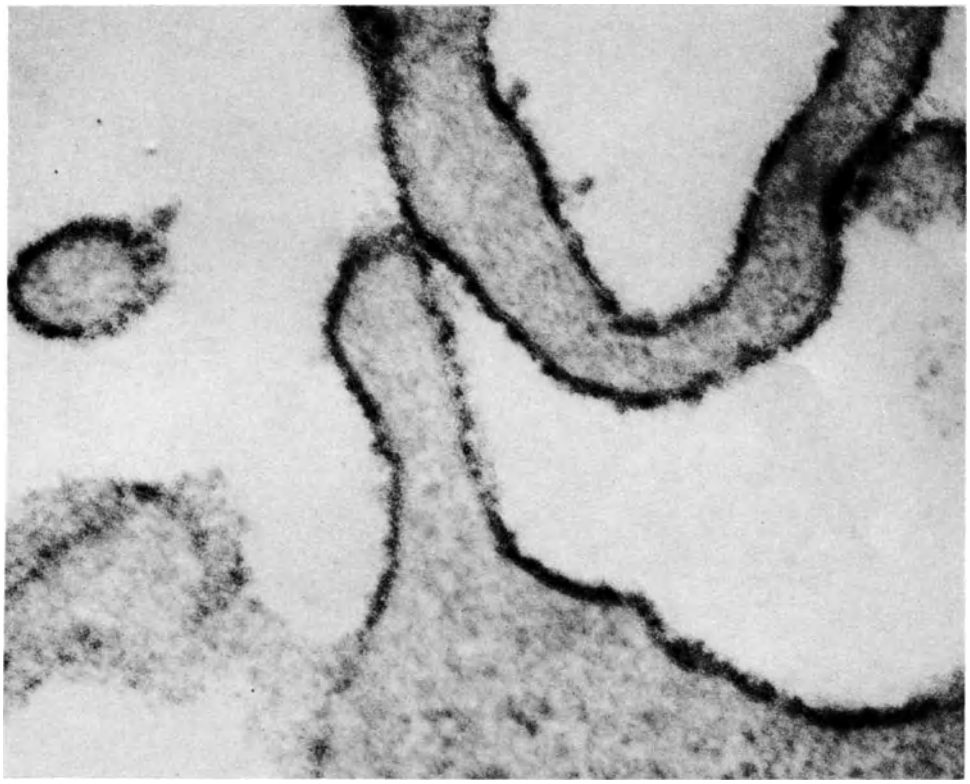

Figure 1.

Surface of peritoneal macrophage stained with ruthenium red. Note the electron dense material adherent to the outer lamina of the cell membrane. Small globular aggregates lie superficial to the main mass of the coat. X 125,000.

Table 1: Loss of Attachment of Aldehyde Fixed Red Cells to Macrophages Following Trypsinization

Sample	Number of experiments	Percentage macrophages with attached red cells
Control	8	74
Trypsinized	6	4
Washed	4	13
Washed + mouse plasma	4	53

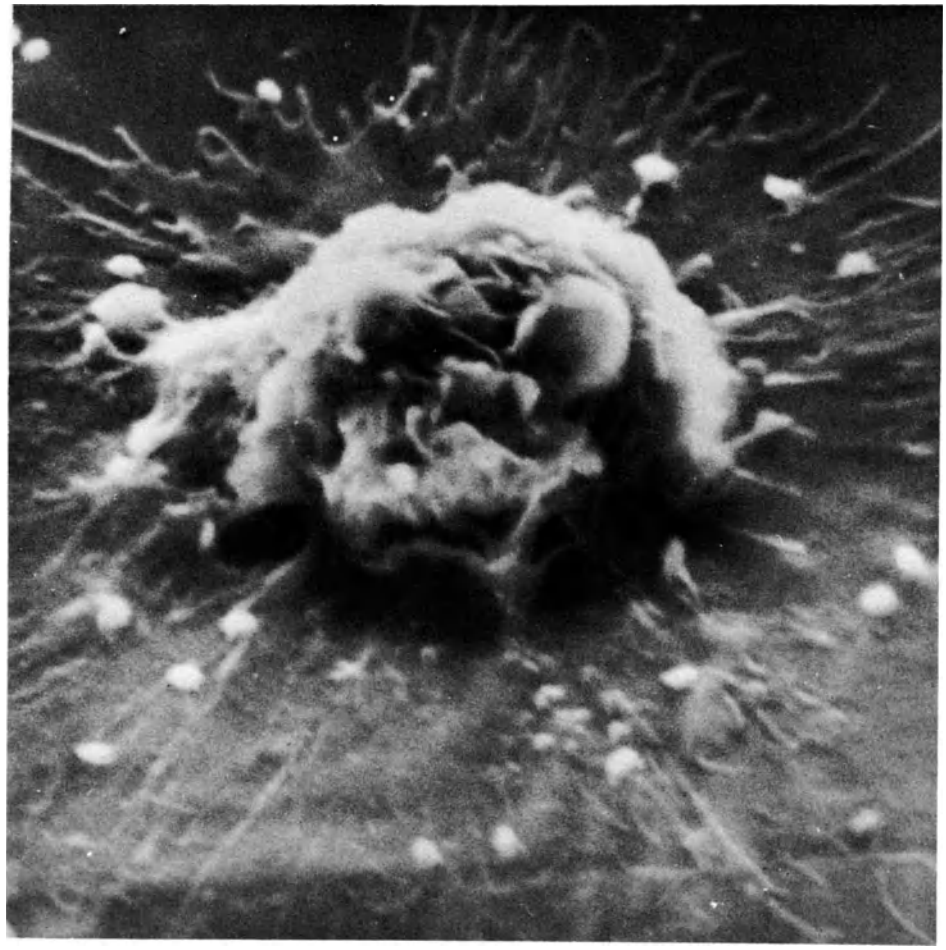

Figure 2.

Peritoneal macrophage fully spread on glass as seen with the scanning electron microscope. Note the numerous fine processes at the periphery of the cell, and the irregular surface of the upper surface. The nature of the small masses of material lying superficial to the spread cytoplasm is uncertain. X 7500.

invaginations, and the globular aggregates of coat material; these may be of significance in phagocytosis.

The process of recognition of aldehyde fixed red cells is largely abolished by trypsin digestion; it may therefore be reasonably inferred that this depends on something which lies superficial to the cell coat, presumably a layer of protein. Trypsinized cells, on the other hand will still stick to glass. This observation it must be emphasized must be contrasted with the common observation that tyrpsinization removes cells from glass. Trypsinization does indeed remove cells from glass, but very often produces severe cellular damage in the process. The gentle trypsinization used in these experiments caused little cellular damage. The results are interpretable in two ways. Either adhesion to glass depends on a layer deep to that which is trypsinized off, i.e. the cell coat; or the cells resynthesize the layer which has been trypsinized off rapidly enough to allow them to stick normally to glass. The latter hypothesis seems less likely in view of the short time (30 minutes or less) needed for firm adhesion to glass.

Summary

The mouse peritoneal macrophage has a prominent and tightly adherent cell coat, well demonstrated by ruthenium red staining. Aldehyde-fixed autologous red cells are recognized at the level of a less adherent presumably more superficial protein layer; adhesion to glass may occur at cell coat level.

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THE FINE STRUCTURE AND PEROXIDASE ACTIVITY OF RESIDENT AND EXUDATE PERITONEAL MACROPHAGES IN THE GUINEA PIG

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The morphology, function, and kinetics of mononuclear phagocytes are usually studied in peritoneal macrophages obtained from experimentally induced peritoneal exudates. Although it has been convincingly demonstrated that most of the mononuclear phagocytes present in peritoneal exudates are monocytes deriving from the peripheral blood, it cannot be entirely excluded that a probably varying proportion of the mononuclear phagocytes present in the peritoneal exudates may be resident peritoneal macrophages, i.e. cells already present in the peritoneal cavity.

In the present study an attempt was made to distinguish between the fine structural characteristics of resident and exudate peritoneal macrophages as well as their cytochemical properties with respect to peroxidase activity.

MATERIAL AND METHODS

Animals

The experimental animals were young female albino guinea pigs weighing 300 to 400 grams.

Isolation of cells

To obtain resident peritoneal macrophages the guinea pigs were intraperitoneally injected, under deep ether anesthesia, with cold sterile physiological saline, which after gentle kneading of the abdomen, was withdrawn from the peritoneal cavity and centrifuged in the cold. Peritoneal exudates were induced by the injection of 30 ml physiological saline into the peritoneal cavity. After various intervals the animals were deeply anesthetized with ether and the peritoneal cavity was washed out with physiological saline. The cell suspensions thus obtained were centrifuged in the cold.

Fixation

For the morphological studies the cell pellets obtained after centrifugation were either resuspended in 1 % phosphate-buffered osmium tetroxide (pH 7.2, 370 mOsm) and fixed for 10 minutes at 4^{o} or similarly fixed at 4^{o} C for 10 minutes in a freshly made mixture of osmium tetroxide and glutaraldehyde, according to Hirsch and Fedorko (1), followed by a postfixation for 15 minutes with uranyl-acetate (1).

For the cytochemical studies the cell pellets were resuspended in 1.5 % glutaraldehyde in 0.067 M cacodylate buffer (pH 7.2) with a final osmolality of 350 mOsm, and fixed for 5 to 10 minutes at 4^{o} C. After incubation, the cells were rinsed in physiological saline and post-fixed for 10 minutes in 1 % phosphate-buffered osmium tetroxide.

Cytochemistry

After fixation, centrifugation and resuspension the cells were washed twice in physiological saline for one minute and then incubated for 60 minutes in an incubation medium (pH 9) for the demonstration of peroxidase activity according to Novikoff and Goldfischer (2). Controls were obtained by incubating the cells in the absence of hydrogen peroxide. In addition, cells were incubated in the complete incubation medium supplemented with 0.02 M 3-amino-1,2,4-triazole or 0.01 M potassium cyanide, or in incubation media containing 2 %

hydrogen peroxide.

Dehydration, embedding and sectioning

Dehydration was performed in a graded series of ethanol solutions, followed by embedding in Epon. Ultrathin sections were examined unstained or stained with lead hydroxide or first uranyl acetate and then lead hydroxide, and studied in a Philips electron microscope EM 300 at 80 kv with a 40 objective aperture.

RESULTS

Resident peritoneal macrophages

The cells obtained from the peritoneal cavities of our animals contained chiefly two types of cells: eosinophilic granulocytes and resident peritoneal macrophages. In contrast to cell suspensions isolated from mouse peritoneal cavities (3) only small numbers of lymphoid cells were present. Only the macrophages will be described here.

1. Fine structure (see Figs. 1 and 2). The macrophages formed a homogeneous population of relatively large cells characterized by an irregularly shaped, elongated, and lobulated nucleus having a relatively large number of pores (4). On one side the nucleus carried prominent bundles of cytoplasmic filaments, the individual filaments having a diameter of about 130 Å after the combined osmium/glutaraldehyde fixation. The cytoplasm of these cells contained well-defined profiles of rough endoplasmic reticulum often arranged in stacks and characteristically located at the same side of the nucleus as most of the filaments. At the other side of the nucleus there was a large Golgi area in which several stacks of Golgi cisternae and numerous, both smooth and small bristle-coated vesicles were distributed around centrally located centrioles. Mitochondria were numerous in the peritoneal resident macrophages and randomly distributed through the cytoplasm, most of them having an elongated shape and well-defined cristae. In addition, the cytoplasm often contained one or two inclusion bodies containing a varying number of electron-dense globules and limited by a single membrane with a width similar to that of the plasma membrane.

These inclusion bodies are probably lysosomal in nature (5). A varying but generally small number of single-membrane-limited electron-lucid vacuoles were present as well as a few multivesicular bodies. The plasma membrane of the resident peritoneal macrophages, with a width of about 100 Å, was clearly trilaminar in both osmium-fixed and in uranyl-acetate post-fixed cells, and revealed the presence of a large number of micro-

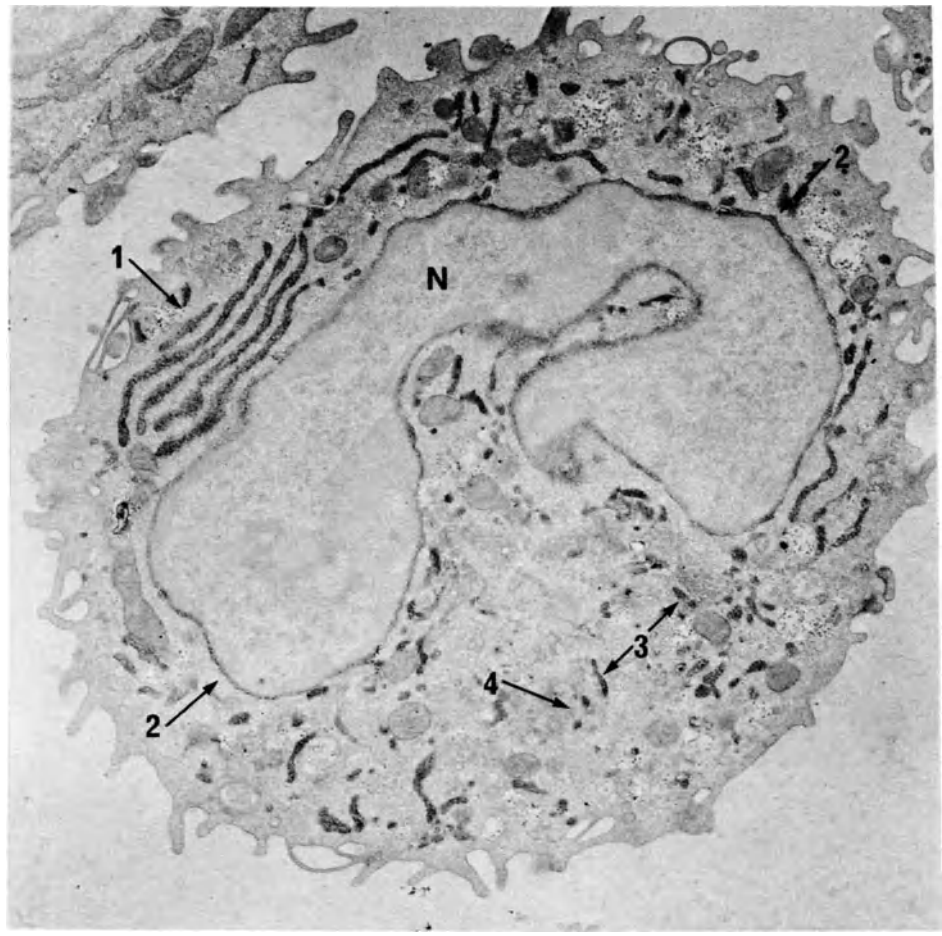

Fig. 1. Resident peritoneal macrophage incubated for the demonstration of peroxidase activity. Reaction product is present in cisternae of the endoplasmic reticulum (1), the nuclear envelope (2), cisternae of the Golgi apparatus (3) and Golgi vesicles (4). Note the irregular shape of the nucleus (N) and the projections of the cell surface. x 14.000.

villous projections. Adjacent to the plasma membrane, a varying number of electron-lucid vacuoles were commonly present, at least some of them suggesting invaginations of the cell surface. The peripheral cytoplasm was devoid of organelles except for a few bristle-coated vesicles.

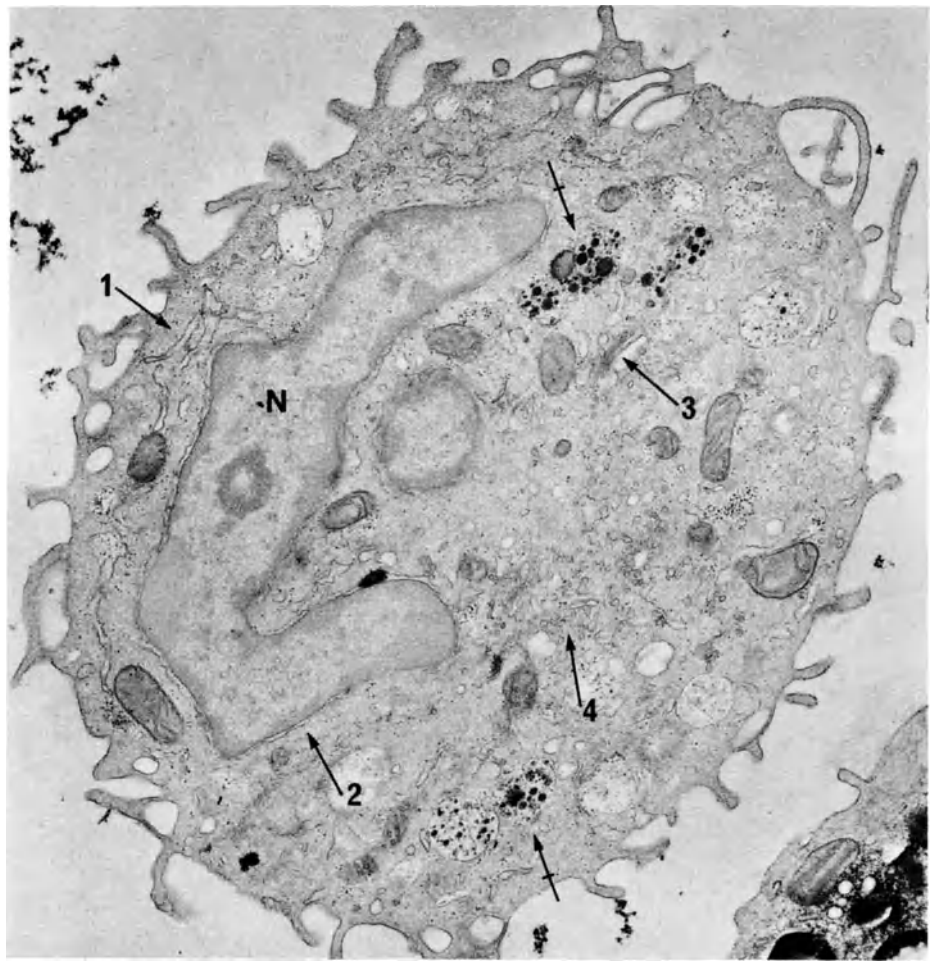

Fig. 2. Resident peritoneal macrophage incubated in a complete medium for the demonstration of peroxidase activity, plus amino-triazole. Reaction product is absent in the cisternae of the endoplasmic reticulum (1), the nuclear envelope (2), cisternae of the Golgi apparatus (3), and Golgi vesicles (4). The cytoplasm contains a number of inclusion bodies (barred arrows) containing electron-dense globules. The cell surface shows numerous projections. x 14.000.

Thanks to the post-fixation in uranyl-acetate, the membranes limiting the various cell organelles clearly revealed a trilaminar structure, its dimensions depending on the type of organelle, the representatives of the exoplasmic space having membranes with greater dimensions than those of the endoplasmic space (6).

2. Cytochemistry (Figs. 1 and 2). After incubation for the demonstration of peroxidase activity, electron-dense reaction product was found in about 90 % of the resident macrophages. It was characteristically present in the cisternae of the rough endoplasmic reticulum, in the nuclear envelope, and in some cisternae of the Golgi apparatus as well as in vesicles present in the Golgi area. Very occasionally, a granule with a diameter of about 0.3 μ containing reaction product was found. Cells incubated up to 180 minutes in the absence of hydrogen peroxide as well as cells incubated in an incubation medium containing 2 % hydrogen peroxide or 0.01 M potassium cyanide showed no reaction product at all. The addition of amino-triazole also resulted in no staining of resident peritoneal macrophages.

Exudate peritoneal macrophages

The exudate elicited by the intra-peritoneal administration of physiological saline consisted predominantly of mononuclear phagocytes and neutrophilic granulocytes. In addition, varying but usually small numbers of lymphocyte-like cells and eosinophilic granulocytes were observed. Depending on the time elapsed after induction of the exudate, the relative proportions of the various types of cells present in the exudates varied considerably.

1. Fine structure (see Figs. 3 and 4). The following description concerns the mononuclear phagocytes present in exudates isolated 16 hours after induction. A minority of these cells satisfied the description of the resident peritoneal macrophages given above, but the predominant type was a cell with a round to oval or horseshoe-shaped nucleus. As compared to the resident peritoneal macrophages, these exudate peritoneal macrophages had only relative scarce short profiles of rough endoplasmic reticulum, and the Golgi apparatus was not very prominent. The cytoplasm contained single-membra-

ne limited vesicles of varying dimensions. Filaments surrounding the nucleus were rarely observed. The cells contained relatively few round to oval mitochondria; the plasma membrane generally had few microvillous projections. Vacuoles adjacent to the plasma membrane occurred relatively infrequently.

2. Cytochemistry (Figs. 3 and 4). After incubation for the demonstration of peroxidase activity, the exudate peritoneal macrophages showed a varying number of highly electron-dense round to oval granules limited by a single membrane and with a diameter of about 0.4 μ, invariably located in the Golgi area. The Golgi apparatus, the scarce elements of the endoplasmic reticulum, and the nuclear envelope contained no product, and no vesicles with reaction product, comparable to those present in the resident peritoneal macrophages, were seen in the cytoplasm of these cells. Cells incubated in the absence of hydrogen peroxide did not contain granules with reaction product. Incubation of the cells in a medium containing amino-triazole did not influence the appearance of distribution of the reaction product, and this also holds for media with a high hydrogen peroxide concentration or containing potassium cyanide.

DISCUSSION

The present results indicate that the mononuclear phagocytes in the unstimulated peritoneal cavity of guinea pigs form a homogeneous population with respect to both fine structure and the distribution of peroxidase activity, and agree in fine structure with the macrophages normally present in the peritoneal cavity of mice (3) and rats (7).

It is noteworthy that the resident peritoneal macrophages in our material did not have the appearance of cells that had undergone any degree of stimulation of their vacuolar apparatus: particles having the structural characteristics of lysosomes (8) were scarce or absent. The macrophages normally present in the peritoneal cavity are known to have a low acid phosphatase acitvity (7, 9).

The results of the cytochemical experiments indicate that the peroxidase activity found in the resident peritoneal macrophages might well be the result of the

peroxidatic activity of catalase: not only was that enzyme activity inhibited in the presence of amino-triazole - a compound generally used as an inhibitor of catalase (10) - but no reaction product developed in the presence of high concentrations of hydrogen peroxide, under which condition catalase has no peroxidatic acti-

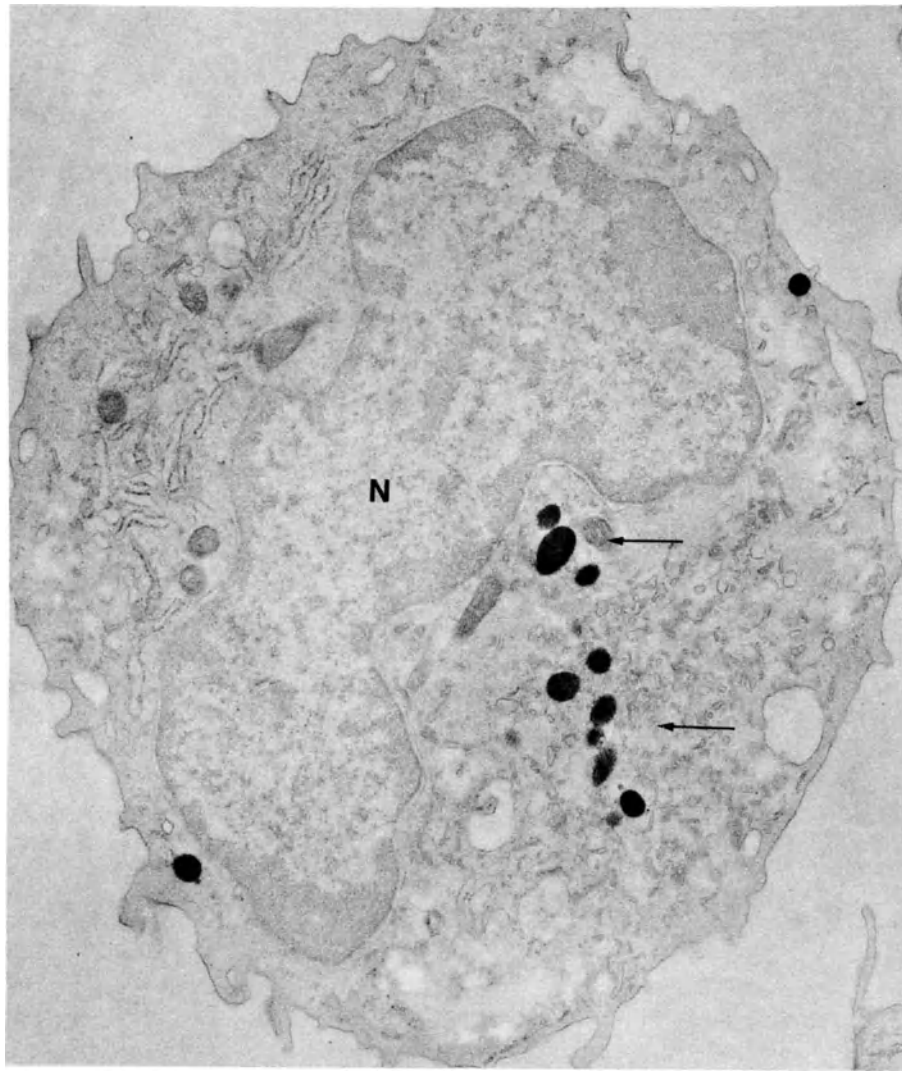

Fig. 3. Exudate peritoneal macrophage incubated for the demonstration of peroxidase activity. In the Golgi area there are granules containing the reaction product (arrows). Note the relative smoothness of the cell surface. x 17.000.

vity (10). In addition, the presence of KCN abolished enzyme activity completely. It is of interest that in these respects the behaviour of the resident peritoneal macrophages resembles that of other tissue macrophages in the guinea pig, such as the Kupffer cells and the alveolar macrophages. In these latter cells peroxidase

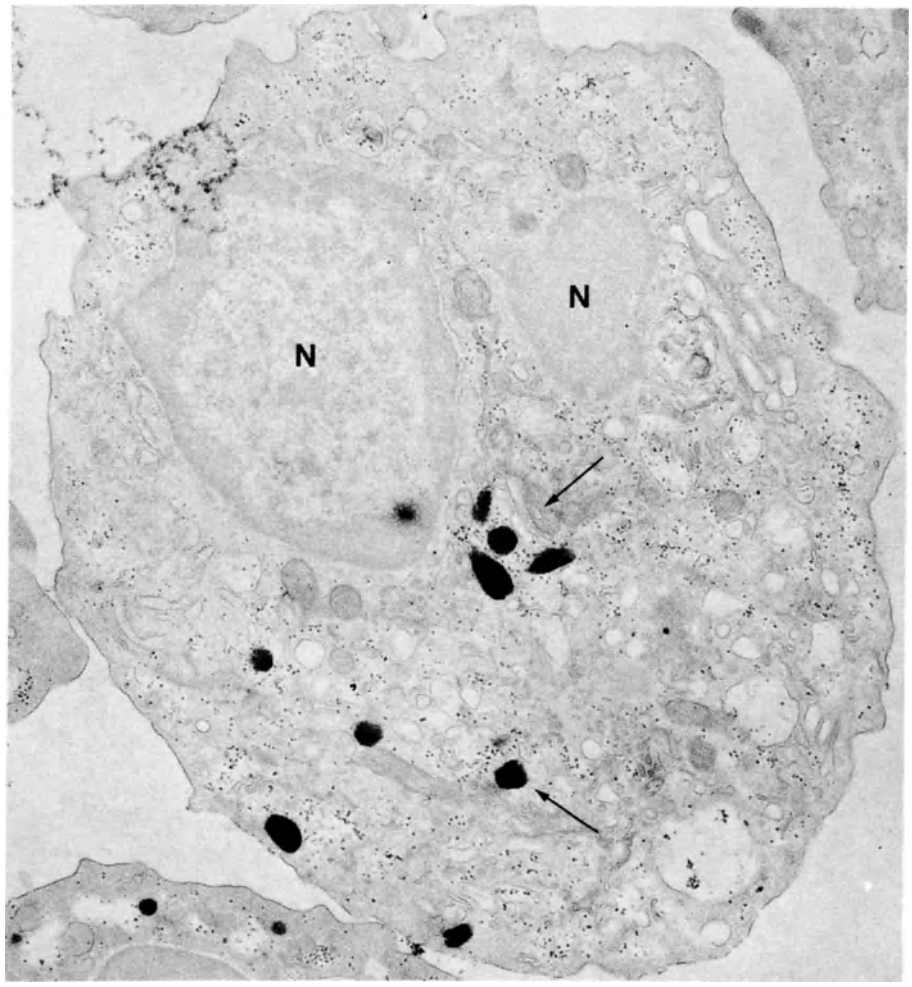

Fig. 4. Exudate peritoneal macrophage incubated in a complete medium for the demonstration of peroxidase activity, plus amino-triazole. In the Golgi area granules containing the reaction product (arrows) are present. The cell surface shows few of the slender projections so characteristic of the resident peritoneal macrophages (compare with Figs. 1 and 2). x 17.000

activity is also located in the endoplasmic reticulum and the nuclear envelope, and can similarly be prevented by adding amino-triazole to the incubation medium (11). In agreement with these findings, Fedorko and Hirsch (3) report that neither peritoneal macrophages nor alveolar macrophages have peroxidase-positive granules. Additional support for the assumption that catalase is responsible for the peroxidase activity of the alveolar macrophages is provided by the biochemical demonstration of high concentrations of catalase in alveolar macrophages reported by Gee et al. (12).

Although the peritoneal exudates provoked by the intraperitoneal administration of physiological saline contained some cells strongly resembling resident peritoneal macrophages, the total cell population of the exudates differed from that normally present in the peritoneal cavity in that the majority of the mononuclear phagocytes in the exudates could easily be distinguished morphologically from the resident peritoneal macrophages. Since it is known that intraperitoneal administration of sterile irritants leads to an accumulation in the peritoneal cavity of monocytes derived from the blood circulation (13), it may be concluded that the mononuclear phagocytes differing from the resident peritoneal macrophages, i.e. the exudate peritoneal macrophages, were in fact cells recruited from the pool of peripheral blood monocytes.

The results of the cytochemical experiments indicate, in agreement with light-microscopical studies on monocytes (14), that these monocytogeneous exudate peritoneal macrophages have granules containing the enzyme peroxidase, a property they share with the azurophilic granules in guinea pig neutrophils (15). The exudate peritoneal macrophages thus appear also to differ cytochemically in a characteristic way from the resident peritoneal macrophages. With reference to certain controversies concerning the origin of the peroxidase-containing granules in monocytes (14, 16), it may be mentioned that it has been demonstrated that the granules in the exudate peritoneal macrophages do not derive from exogeneous sources such as ingested neutrophils (17). This is in agreement with the assumption that they are produced by the promonocytes of the bone marrow (3), which too have peroxidase-positive granules (18).

The results of the present study indicate that re-

sident peritoneal macrophages can indeed be distinguished both morphologically and cytochemically from the monocytogeneous exudate peritoneal macrophages.

Furthermore, peritoneal exudates have been shown to contain both resident peritoneal macrophages and exudate peritoneal macrophages and thus do not form a homogeneous population with respect to the mononuclear phagocytes, although both cell types show a similar phagocytic activity, with retention of their respective morphological and cytochemical characteristics (5).

Finally, as mentioned above, monocytes are considered to be the precursor cells of sessile tissue macrophages (19). It seems pertinent to this consideration that the results obtained in the present study indicate that, in addition to the characteristic differences existing between the resident peritoneal macrophages on the one hand and exudate peritoneal macrophages on the other, transitional forms between these two types of cells have thus far not been observed in our material.

SUMMARY

1. Resident peritoneal macrophages, i.e. mononuclear phagocytes normally present in the peritoneal cavity, can be distinguished morphologically and with respect to the distribution and nature of their cytochemically demonstrable peroxidase activity from monocytogeneous peritoneal macrophages occuring in experimentally induced exudates. Peritoneal exudates contain both types of cells.

2. Transition forms between resident peritoneal macrophages and exudate peritoneal macrophages were not observed.

ACKNOWLEDGEMENTS

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KINETICS, CYTOCHEMISTRY AND DNA-SYNTHESIS OF BLOOD MONOCYTES IN MAN*

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There is now a great deal of evidence to prove that monocytes are formed in the bone marrow. After circulating for a certain time these cells leave the vascular tree and migrate into the tissue. Here they give rise to tissue macrophages (1-12).

The intravascular part of the life cycle of the macrophages is particularly suited to experimental observation as monocytes can be isolated easily. Cellkinetic studies using ^{3}H-thymidin labeling have shown that the intravascular period of the monocytes is relatively short, and it has been estimated to last about one day (13, 14). The data given by this method, however, are influenced by several factors which cannot be determined exactly. These results, therefore, only give an approximate account of the actual cellkinetic processes.

It was shown by our experiments that monocytes can be stably labeled with tritiated diisopropylfluorophosphate. Therefore the autotransfusion technique of monocytes labeled in vitro could be used to examine this problem. This method has the advantage that apart from the intravascular half-time of disappearance of the labeled cells the total blood monocyte pool and the monocyte turnover rate can be determined as well.

In this paper the cytokinetic data obtained from normal

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subjects and those with monocytosis are related to cytological, cytochemical and functional parameters of monocytes. By this means information is gained about monocyte precursors on the one hand and the proliferative potential of the macrophages on the other.

MATERIALS

Autotransfusion of ^{3}H-DFP labeled monocytes was performed on 6 patients with normal monocyte count and on 9 patients with monocytosis caused by the following diseases: chronic infections (4 cases), malignant tumors (3 cases), miliary lupoid (1 case), cyclic neutropenia (1 case).

Cytological, cytochemical and ^{3}HTDR-incorporation experiments were performed on 10 normal subjects and 11 patients with monocytosis. 8 patients suffered from chronic infections and 3 from bronchial cancer. Patients with obvious splenomegaly and acute infections are not included in this paper.

METHODS

Determination of cytokinetic parameters. The experiment was begun at 7:30 a.m. The patients had not eaten since the previous day. A sample of about 500 ml blood was taken in a sterile, pyrogen-free plastic bag containing about 80 ml ACD. 750 µCi triated diisopropylfluorophosphate (^{3}H-DFP, spec. act. 4.8 Ci/mM, Radiochemical Centre Amersham, England) dissolved in 0.15 ml propylen glycoll, was added. After an incubation period of 1 hour at room temperature the autotransfusion was performed over a period of 10-15 minutes. The number of the infused labeled monocytes was calculated from the amount of blood transfused, the average monocyte count during transfusion and the labeling index.

At suitable intervals after transfusion, venous blood samples were taken for preparing leukocyte concentrate smears (15). After photographic processing the preparations were stained by GIEMSA.

The labeling index of the circulating monocytes was determined by counting 2,000 monocytes in autoradiographs. The half-time of disappearance ($T\frac{1}{2}$) of the cells was determined by plotting the labeling indices against time after transfusion in a semilogarithmic coordinate system.

The size of the total blood monocyte pool (TBMP) was calculated according to the principal of dilution in terms of 1 Kg

body-weight. The data for the circulating monocyte pool represent the calculated number of monocytes in the blood volume (body-surface in square meters x 2,680 (16)).

The monocyte turnover rate (MTR) was calculated using a formula analogous to that which ATHENS et al. (17) used for the neutrophil turnover rate: MTR = TBMP x $\ln 2/T\frac{1}{2}$.

Skin window experiments according to REBUCK (18). On some of the patients 6 hours prior to the autotransfusion, skin-areas of about 3 x 5 mm were abraded from the epidermis lateral from the tibia. The abraded area was closed with a 24 x 24 mm coverslip after adding a drop of diphtheriatoxoid (Behring-Werke, Marburg, W. Germany). During autotransfusion the coverslips were changed for fresh ones. These were removed after an interval of between 6 and 48 hours.

^{3}H-thymidin (^{3}HTDR) incorporation of monocytes in vitro. 2 µCi/ml ^{3}HTDR was added to the mixture for the preparation of leukocyte concentrates. After a sedimentation time of 30 minutes, leukocyte concentrate smears were prepared, fixed in absolute methanol, and processed for autoradiography. The exposure time was about one week. In the stained autoradiographs 1,000 monocytes were counted to determine the labeling index.

Cytochemical experiments. Leukocyte concentrate smears of venous blood were submitted to the following cytochemical reactions: 1) Naphthol-AS-D-chloroacetate-esterase according to the method described by LEDER (19). The preparations were incubated twice (20 and 30 minutes). 2) Naphthol-AS-acetate-esterase using the method described by LÖFFLER (20). In each preparation 300 monocytes were analyzed according to their nucleus morphology and the intensity of the reaction. For the naphthol-AS-acetate-esterase 3 degrees of reaction intensity were distinguishable (0; I; II) and for the naphthol-AS-D-chloroacetate-esterase 4 degrees of reaction intensity (0; I; II; III;). The "Activity Index" was calculated by adding the products of 100 monocytes and their degree of reaction intensity.

RESULTS

Labeling of Leukocytes with ^{3}H-DFP

In the autoradiographs, prepared from blood samples taken from the plastic bag after incubation with ^{3}H-DFP, 96-100 % of the monocytes were clearly distinguishable from the background as labeled (Fig. 1). The grain count was similar to

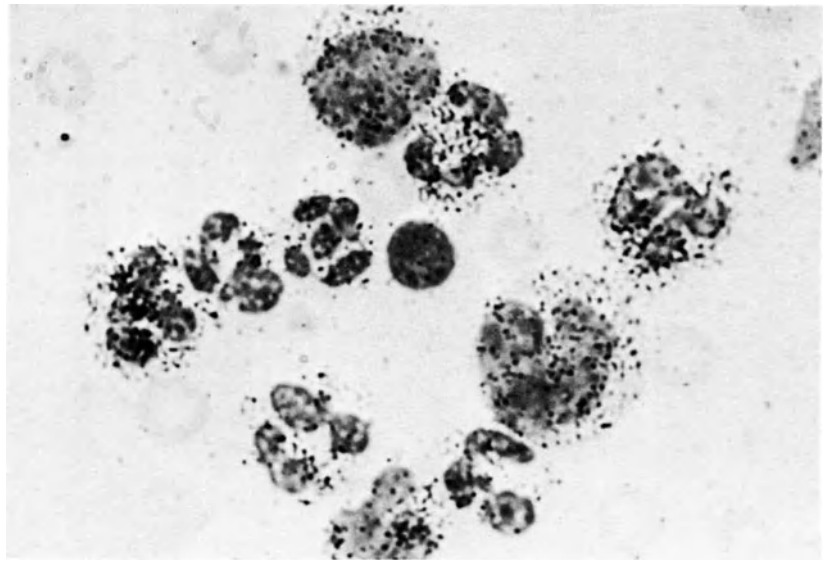

Fig. 1.- Labeled human blood monocytes and neutrophils after incubation of blood with ^{3}H-diisopropylfluorophosphate.

that of the labeled neutrophils. In 8 experiments it ranged from 43-82 per cell with an average exposure time of 81 days. With this exposure time the grain count of lymphocytes did not differ significantly from the background.

Intravascular Behaviour and Fate of the Transfused ^{3}H-DFP Labeled Monocytes

In all the experiments performed, the labeling indices of the circulating monocytes after transfusion gave basically similar graphs to that shown in Fig. 2. Within the first 2 hours after the completion of the autotransfusion the labeling indices declined rapidly by a mean of about 40 %. After this the decline of the labeling index was slower and followed an exponential function.

A loss of grain count of the labeled monocytes was detected which was relative to the circulation time. But this loss amounted only to 0.04 % of the initial labeling per 10 hours and therefore did not influence the cytokinetic results obtained by autoradiography.

In the skin window experiments labeled skin window macrophages could be detected (Fig. 3) from about the sixth hour after autotransfusion. These showed a similar grain count as

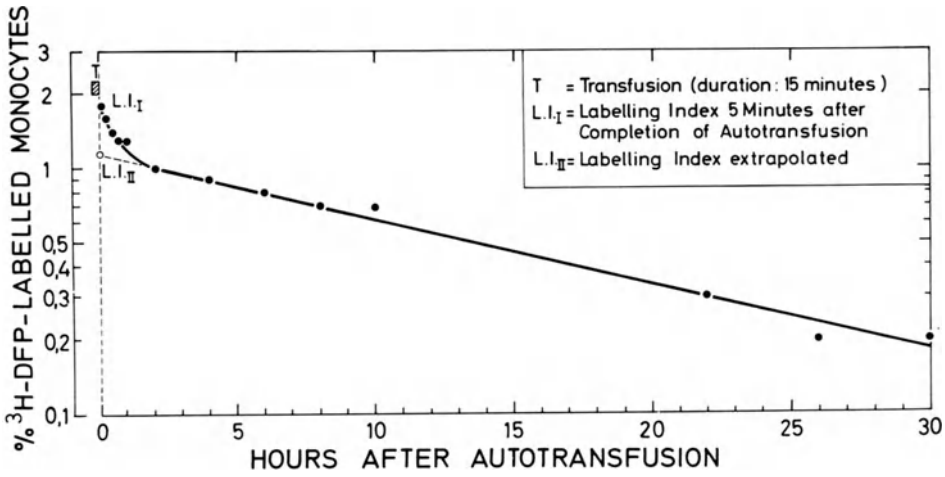

Fig. 2.- Labeling index of circulating monocytes after autotransfusion of ^{3}H-diisopropylfluorophosphate labeled monocytes.

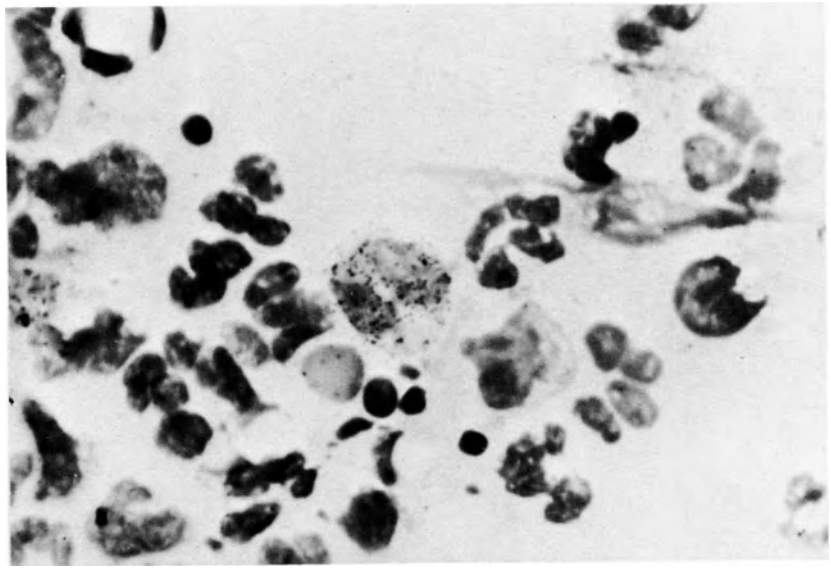

Fig. 3.- Labeled skin window macrophage on a coverslip removed 10 hours after autotransfusion of labeled monocytes.

the transfused labeled monocytes. Besides this, skin window macrophages were observed, which contained round radioactive areas in the cytoplasma. It may be assumed that these cells

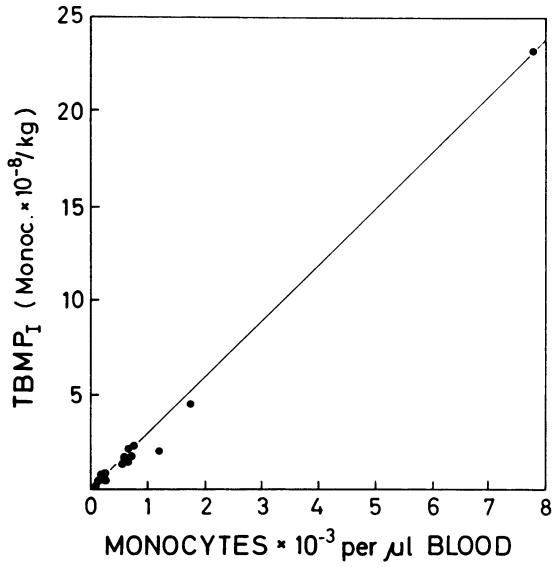

Fig. 4.- Total blood monocyte pool I plotted as a function of the monocyte count in the blood.

had incorporated radioactive material by phagocytosis.

Total Blood Monocyte Pool (TBMP)

The determination of TBMP was problematic because the graph of the labeling indices was divided into 2 parts. 2 values were therefore calculated: $TBMP_I$, using the labeling index 5 minutes after the completion of autotransfusion ($L.I._I$; Fig. 2) and $TBMP_{II}$, using $L.I._{II}$ obtained by producing the second part of the curve to T_o.

The mean of $TBMP_I$ was 3.9 times as great as the circulating monocyte pool (CMP). The value of $TBMP_{II}$ was larger than that of $TBMP_I$ by a mean of about 65 %. As no sickness-specific deviations between $TBMP_I$ and $TBMP_{II}$ could be detected, only the values of $TBMP_I$ and MTR_I calculated from it are given in this paper. As shown in Fig. 4 $TBMP_I$ increased approximately proportionally to the monocyte count in the blood circulating. In none of the cases examined was there a significant deviation from this.

Half-Time of Disappearance ($T\frac{1}{2}$)

In all the patients examined the loss of the labeled cells

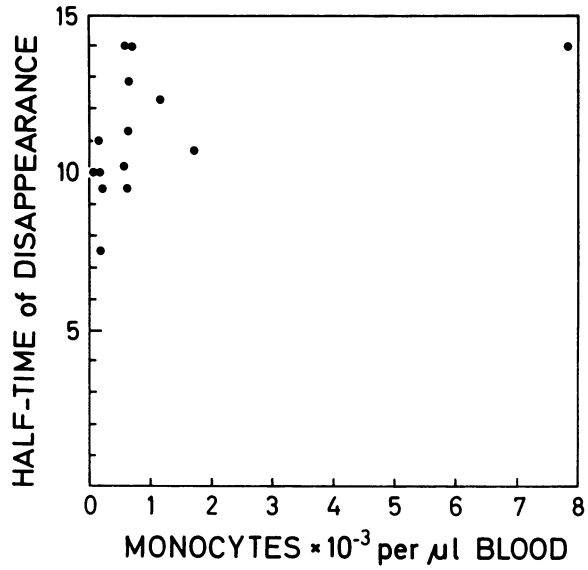

Fig. 5.- Half-time of disappearance of autotransfused ^{3}H-diisopropylfluorophosphate labeled monocytes in patients with normal monocyte counts and with monocytosis.

in circulating blood followed an exponential function from the second hour after completion of the autotransfusion (Fig. 2). The $T_{\frac{1}{2}}$ ranged from 7-11 hours in patients with normal monocyte counts (Fig. 5). In some of the patients with monocytosis $T_{\frac{1}{2}}$ was extended to a maximum of 14 hours.

Monocyte Turnover Rate (MTR)

In patients with normal monocyte counts MTR_I was about 0.5×10^7 per Kg body weight per hour. MTR increased approximately proportionately with the monocyte count in the blood (Fig. 6). In a patient with extreme monocytosis of about 7,840 monocytes per µl blood MTR_I reached 10.7×10^7 per Kg per hour.

Morphological Findings

The blood monocytes could be divided into 3 groups according to their nucleus morphology:
1) Monocytes with lobed nucleus which showed a fine chromatin structure. In PAPPENHEIM-stain the cytoplasma appeared as greyishblue and often contained fine azurophilec granules.
2) "Intermediate forms", whose nuclei were only slightly lobed and showed a more dense chromatin structure. The cytoplasma was

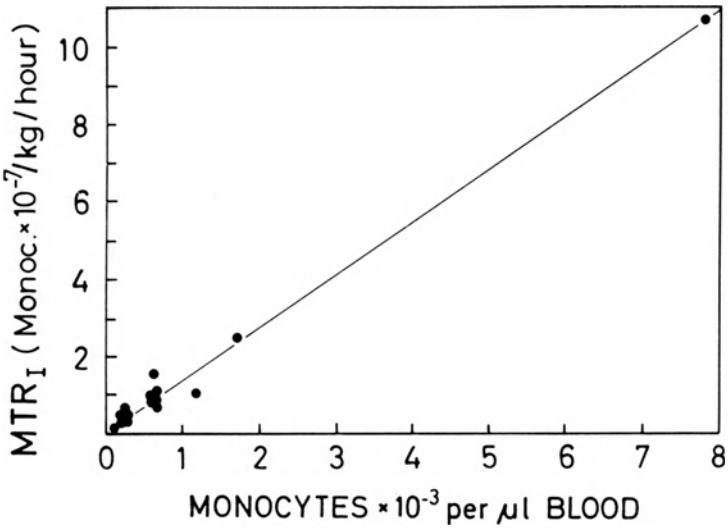

Fig. 6.- Monocyte turnover rate I plotted as a function of the monocyte count in the blood.

more basophilic than in the first group of monocytes.
3) Monocytes with oval or round nucleus with a dense chromatin structure often containing nucleoli. The cytoplasma showed a marked basophilia and in some of the cells it contained promyelocyte like coarse granules.

In the 10 normal subjects, whose mean monocyte count was 370±110 per µl blood the monocytes with lobed nucleus were the most frequent: 58.1 % ± 10.1 %. The proportion of intermediate forms was somewhat less: 34.1 % ± 8.3 %. Monocytes with oval nucleus occurred relatively seldom: 8 % ± 2.6 % (Table 1). The number of the 3 monocyte forms increased approximately in proportion to the monocyte count in the blood (Fig. 7). The monocytes with oval nucleus only showed a clear increase with monocyte counts of over 600 per µl blood. The rate of increase of the 3 types of monocytes, however, showed clear differences: the increase of monocytes with oval nucleus was the highest and that of those with lobed nucleus the lowest. For this reason the proportion of the 3 forms shifted in favour of monocytes with oval nuclei parallel to the increase of the monocyte level in the blood.

Cytochemical Findings

The enzyme pattern of the monocytes with lobed nucleus was characterized by a high activity of naphtol-AS-acetate-esterase

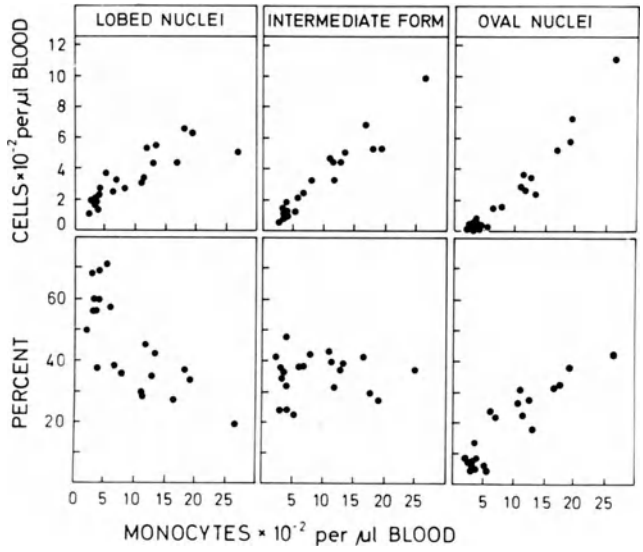

Fig. 7.- Results of the differentiation of blood monocytes according to their nucleus-morphology at different blood monocyte concentrations.

Monocyte Turnover Rate (Monoc. × 10^{-7}/kg/hour)			~ 0.5	~ 1-2	~ 2-4
Monocyte Count (per μl blood)			200-500	1000-2000	2000-3000
Morphology of Nuclei	lobed (%)		58	36	29
	interm. (%)		34	38	34
	oval (%)		8	26	37
^{3}HTDR-Labelling Index	lobed (%)		0.05	0.28	1.65
	interm. (%)		0.54	1.27	4.28
	oval (%)		3.28	1.50	4.87
Naphthol-AS-D-Chloroacetate Esterase	lobed	(enzyme activity indices)	26	43	62
	interm.		53	94	108
	oval		128	163	181
Naphthol-AS-Acetate-Esterase	lobed		141	163	148
	interm.		134	128	138
	oval		73	50	77

Table 1.- Blood monocyte concentration, monocyte nucleus morphology, enzyme pattern and ^{3}HTDR incorporation in relation to the monocyte turnover rate.

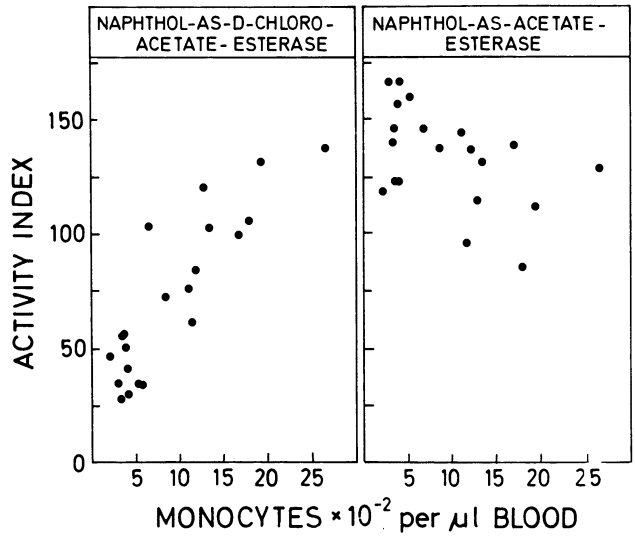

Fig. 8.- Activity indices of naphthol-AS-D-chloroacetate-esterase and naphthol-AS-acetate-esterase in blood monocytes as a function of blood monocyte concentration.

and a low activity of naphthol-AS-D-chloroacetate-esterase (Table 1). In comparison with these cells monocytes with oval nucleus showed a higher naphthol-AS-D-chloroacetate-activity and a lower naphthol-AS-acetatesterase activity. The cytochemical findings of the intermediate forms lay between these. The activity indices of the naphthol-AS-D-chloroacetate-esterase increased in all the 3 monocyte forms parallel to the degree of monocytosis. With naphthol-AS-acetate-esterase no differences were apparent. Fig. 8 shows the enzyme indices in the total blood monocyte population plotted against the blood monocyte concentration. The correlation was clearly positive with the naphthol-AS-D-chloroacetate-esterase and slightly negative with naphthol-AS-acetate-esterase.

^{3}HTDR-Incorporation of Monocytes in Vitro

In 9 normal subjects with monocyte counts of 350 ± 90 per µl blood labeling indices of 0.43 % ± 0.02 % were determined. In 6 patients with monocyte counts in the region of 650-1310 per µl blood the mean labeling index was 0.9 %. When the monocyte count exceeded 1,500 per µl blood, the rate of the labeling index became higher (Fig. 9). The labeling indices of the monocytes with oval nucleus proved to be the highest, whereas those of the forms with lobed nuclei was the lowest (Table 1).

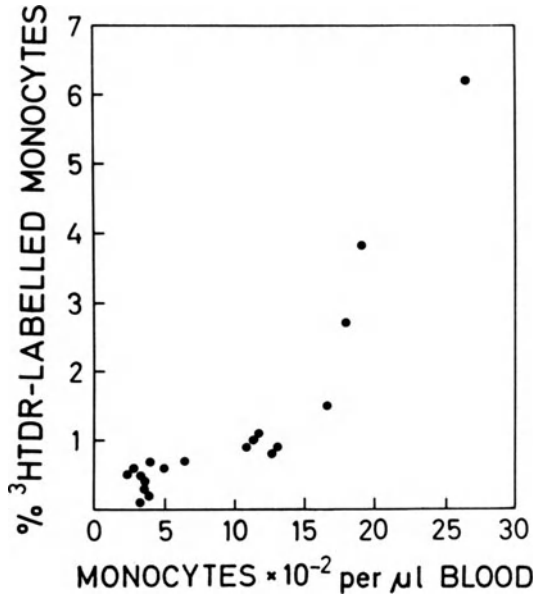

Fig. 9.- ^{3}HTDR-labeling index of blood monocytes as a function of blood monocyte concentration.

DISCUSSION

KURTH and ATHENS (21) found a monocyte labeling index of about 20 % after incubation of blood with ^{3}H-DFP. The considerably higher labeling index of 96- 100 % found in these experiments can be ascribed to the fact that the specific activity of the ^{3}H-DFP used was higher by a factor of 10 and moreover the concentration of DFP in the incubation medium was higher by a factor of 200. It is not clear, however, if the labeling affects monocyte kinetics. It has been proved that neutrophil kinetics is not influenced by DFP-concentrations as used in our experiments (22). The stability of ^{3}H-DFP-labeling proved to be sufficient for the study of monocyte kinetics and the fate of the cells after their emigration from the vascular system.

5 minutes after the completion of the autotransfusion which lasted about 15 minutes it was found that the labeled monocytes had already dispersed in a pool (TBMP), whose size exceeded that of the circulating monocyte pool (CMP) by a factor of about 3.9. It can be deduced from these findings that the circulating monocytes interchange with a second intravascular monocyte pool which does not circulate and which can be viewed as a marginal monocyte pool (MMP) analogous to the findings concerning the neutrophil system. CMP and MMP_I form toge-

ther $TBMP_I$ which can be considered as one pool for cytokinetic purposes because of the rapid cell interchange.

The rapid initial slope of the graph which shows a loss of labeled cells of about 40 % during the first 1-2 hours after the completion of transfusion cannot be interpreted certainly. There are 2 main processes which must be considered: 1) The rapid elimination of monocytes which were damaged during the labeling procedure. 2) The final achievement of a steady state equilibrium of the transfused monocytes in the vascular system. When T_0, extrapolated from the second part of the curve ($L.I._{II}$), was used for the calculation of the pool sizes ($TBMP_{II}$, MMP_{II}) and MTR_{II}, only the absolute values for TBMP and MTR_{II} increased by about 65 %, but these data did not result in any basically new points of view.

Later in the experiment the labeled monocytes circulating in the blood disappeared exponentially. These results were consistent with the findings obtained using the ^{3}HTDR-method (13, 14), namely that monocytes leave the vascular tree randomly. The half-time of disappearance ranged from 7-14 hours. It was, therefore, considerably shorter than deduced from the ^{3}HTDR-experiments (13, 14).

Because of the large number of monocytes marginating and the short half-time of disappearance, unexpectedly high values for MTR resulted. MTR_I amounted to 0.5 x 10^7/Kg/hour in patients with normal monocyte counts and increased in patients with monocytosis in proportion to the blood monocyte concentration. It therefore seems possible to make certain conclusions about the value of MTR simply by determining the monocyte count in the blood. This is not true, however, for patients with acute infections or with clear splenomegaly (23).

It is improbable that the monocyte population undergoes considerable quantitative changes during the passage through the vascular system because of the short half-time of disappearance, the low ^{3}HTDR labeling index and the negligible recirculation of monocytes through the ductus thoracicus (24). It can therefore be assumed that MTR represents approximately the influx rate of the monocytes from the bone marrow into the blood.

Further it may be assumed that the population of blood monocytes shows similar properties as the monocytes flowing into the blood and those emigrating into the tissue. The results of the cytological, cytochemical and ^{3}HTDR experiments (Table 1) support the following final conclusions: With a normal monocyte influx rate of about 0.5 x 10^7/Kg/hour the monocyte population which leaves the bone marrow is composed of about 60 % monocytes

with lobed nucleus, 30 % intermediate forms and 10 % monocytes with oval nucleus. All 3 cell forms are capable of DNA-synthesis. Because of the relatively high ^{3}HTDR labeling index the monocytes with oval nucleus must be regarded as immature forms, whereas the monocytes with lobed nucleus must be regarded as the most mature cell form because of the very low labeling index. The enzyme pattern of the monocytes with oval nucleus which is marked by a high naphthol-AS-D-chloroacetate-esterase activity and low naphthol-AS-acetate-esterase activity proved to be identical with monocyte precursor cells in the bone marrow which LEDER (9) described.

The proportion of immature monocyte forms appearing in the blood increased parallel to the blood monocyte concentration. While at normal monocyte influx rates of 0.5×10^{7}/Kg/hour the ratio of lobed to intermediate to oval forms was about 6:3:1 it changed to 3:3:4 when the influx rate increased to about 3×10^{7}/Kg/hour. This shift in favour of immature forms led to a marked increase in DNA-synthesizing monocytes, a rise of naphthol-AS-D-chloroacetate-esterase activity in the total blood monocyte population. As cells leave the blood randomly and as the increase of immature monocyte forms in the blood did not change $T\frac{1}{2}$ significantly, it may be assumed that the monocyte population emigrating into the tissue possesses similar qualitative characteristics to the blood monocytes.

SUMMARY

Monocytokinetic studies were performed on human subjects using autotransfusion of ^{3}H-DFP labeled monocytes. The monocyte labeling index in the circulating blood was determined by autoradiography. The results gave evidence of the existence of a rapid intravascular interchange between circulating and marginating monocytes. The marginal monocyte pool was calculated to exceed the circulating pool by a factor of about 4. The labeled monocytes disappeared from the blood exponentially. In normal subjects the half-time of disappearance ranged from 7 to 11 hours. The turnover rate amounted to about 5×10^{6} monocytes per hour per Kg body weight. In patients with monocytosis the monocyte turnover rate increased in proportion to the blood monocyte count. Parallel to this a greater amount of immature monocytes appeared in the blood. These cells had oval or round nuclei and showed higher ^{3}HTDR-labeling indices, higher naphthol-AS-D-chloroacetate-esterase and lower naphthol-AS-acetate-esterase activities than mature monocytes with lobed nuclei.

ACKNOWLEDGEMENT

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DIFFERENTIATION OF MACROPHAGE ANTIGEN(S) IN MOUSE BONE MARROW AND BUFFY COAT CULTURES

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Kinetic studies have demonstrated that the mononuclear phagocytic cells or macrophages of serous cavities, lung alveoli and connective tissue originate from precursor cells (promonocytes) in the bone marrow (1-3). These cells give rise to the peripheral blood monocytes, which constitute a mobile pool capable of differentiating further into macrophages at different sites.

Mature mouse macrophages in the peritoneal cavity and lung alveoli possess membrane antigen(s), which can be detected by cytotoxicity tests and immunofluorescent staining using anti-macrophage serum (AMS) (4). We have studied the differentiation of this antigen in the mononuclear phagocyte system. The results indicate that the bone marrow precursors do not contain the antigen, and that the antigen accumulates in macrophage precursors at the monocytic stage.

METHODS

AMS was obtained from rabbits immunized with mouse macrophages. Peritoneal macrophages from CBA mice were cultured in the presence of conditioned medium from L-cell cultures (5), and harvested after two weeks, at which time the number of macrophages was approximately 15 times the initial number, and no lymphocytic cells were present. Immunization was performed according to the method of Unanue (6).

Bone marrow cells were obtained from femurs of adult CBA mice. Blood was collected by heart puncture, and buffy coat cells were separated by sedimentation in 'Plasmagel' (7). For culture, 5-10 x 10^6 bone marrow or buffy coat cells were plated in 35 mm plastic

dishes provided with five small cover slips. The cells were cultured in BME with 10% calf serum in humidified atmosphere of 5% CO_2 in air. Cover slips were harvested at different time intervals and stained with May-Grünwald-Giemsa (MGG) or with immunofluorescent methods.

In cytotoxicity tests cell suspensions were incubated for 30 min in the presence of various dilutions of AMS and complement. Cytotoxicity was determined by dye exclusion using trypan blue. If the cells were to be cultured after the treatment, the suspension was washed with the culture medium and plated in dishes. Controls were treated with normal rabbit serum and complement.

Fluorescent antibody staining was performed by the indirect technique by using FITC-conjugated anti-rabbit-Ig and acetone fixed cover slip cultures.

RESULTS

Specificity of AMS

Without absorptions, the AMS used in these experiments had a cytotoxic titer of 1:320 against peritoneal exudate cells, but a considerable lymphocytotoxic and hemolytic activity was also present (Table 1). Absorption of AMS with mouse RBC or with mouse lymph node cells removed cytotoxicity to the respective target cells, without significantly affecting the cytotoxic titer against macrophages. After absorption with both mouse RBC and lymph node cells, an apparently monospecific antiserum was obtained.

Table 1. Specificity of AMS before and after absorptions[+]

Absorption	Target cell[++]					
	PEC	CM	LNC	SC	ThC	RBC
Unabsorbed	320	640	40	80	<5	320
Absorbed with RBC	160	ND	20	ND	ND	<5
Absorbed with LNC	160	ND	<5	ND	ND	160
Absorbed with RBC & LNC	80	ND	<5	ND	ND	<5

[+]Figures refer to highest dilution of AMS lysing 50% of target cells in cytotoxicity tests.

[++]PEC = peritoneal exudate cells, CM = cultured macrophages, LNC = lymph node cells, SC = spleen cells, ThC = thymus cells, RBC = red blood cells, ND = not done.

After immunofluorescent staining, using either unabsorbed or lymph node cell absorbed AMS, mouse peritoneal and alveolar macrophages showed a strong membrane fluorescence. Mouse fibroblasts and lymphocytes were negative. In double diffusion precipitation tests using absorbed AMS, a single precipitation line was obtained with homogenates of peritoneal exudate cells and cultured macrophages, but not with lymph node, thymus or fibroblast homogenates.

Effect of AMS on Bone Marrow and Buffy Coat Cells

To demonstrate whether bone marrow and buffy coat contained cells possessing macrophage-specific antigen(s), cytotoxicity and immunofluorescence tests were applied. Unabsorbed AMS at 1:10 dilution lysed approximately 40% of bone marrow cells and 80% of buffy coat cells. In bone marrow the cytotoxic effect was mainly directed against RBC precursors, and in buffy coat against lymphocytes. Accordingly, absorption with RBC and lymph node cells abolished most of this cytotoxicity. In immunofluorescence tests no cells with clear membrane fluorescence could be detected, but the nonspecific staining of granulocytes and their precursors caused difficulties in the evaluation of the tests.

As a further test to detect whether cytotoxicity was directed against macrophage precursors, bone marrow and buffy coat cells were plated for culture after treatment with various concentrations of AMS and complement. Even when treated with unabsorbed AMS at 1:10 dilution, bone marrow cells gave rise to control numbers of macrophages during subsequent culture. After similar treatment, no macrophages emerged in cultures of peritoneal exudate cells. When buffy coat cells were treated in the same way, the number of macrophages in 4-day-old cultures was approximately 10% of the number in control cultures. Serial dilutions of unabsorbed AMS, and the use of lymph node cell absorbed AMS, revealed that the cytotoxic effect of AMS against macrophage precursors in buffy coat was smaller than against peritoneal exudate cells.

Effect of AMS on Cultured Bone Marrow and Buffy Coat Cells

In immunofluorescent staining, cells showing membrane fluorescence were detected both in bone marrow and buffy coat cultures. In buffy coat cultures the number of positive cells increased more rapidly, and in both types of cultures the number of positive cells correlated to the number of cells scored as macrophages in MGG stained cover slips.

When bone marrow cultures, which were 3 hours to 5 days old, were incubated with unabsorbed AMS at 1:10 dilution in the presence of complement, all cells with macrophage-like morphology were lysed.

However, when these cultures were washed after the treatment, and recultured, macrophages again appeared in the cultures. At three hours this had no significant effect on the formation of macrophages, and in older cultures decreasing numbers of macrophages were found (Table 2). Following AMS treatment on day 5, only occassional macrophages were found 4 days later. When one-day-old buffy coat cultures were similarly treated with AMS and recultured, no macrophages appeared in the cultures.

Table 2. Effect of AMS and complement treatment on the formation of macrophages in vitro

Cells cultured	Time of treatment (hours)	Number of macrophages 96 hours later (% of control)
Bone marrow	3	93
	24	41
	72	20
	72+	1
	120	2
Buffy coat	24	0

+10 μCi/ml ^{3}H-TdR in the medium before AMS treatment.

The experiments with bone marrow cultures indicated that some precursor cells which were not affected by AMS, persisted for at least three days in vitro. To study whether this cell population was proliferating in the cultures, bone marrow cells were cultured for three days in the presence of 10 μCi/ml of tritiated thymidine in order to block cell division. When these cultures were thereafter treated with AMS and complement, practically no macrophages were found 4 days later (Table 2).

CONCLUSIONS

The results indicate that macrophage precursors in the bone marrow do not contain detectable amounts of membrane antigen(s) which seem to be characteristic to mature macrophages. Neither could the antigen be detected in a part of blood monocytes, the majority of the monocytes, however, possessing the antigen. The amount of the antigen in blood monocytes appeared to be smaller than in mature macrophages. Thus, the membrane antigen seems to be formed during the monocyte stage of macrophage differentiation.

In cultures of bone marrow and buffy coat cells, the antigen is formed in parallel with the morphological differentiation of macrophages. Proliferating precursor cells were found in bone marrow but not in buffy coat cultures.

The macrophage membrane antigen seems to be useful in the characterization of the cellular differentiation in the mononuclear phagocyte system, and its presence could be used as a marker in the identification of cells belonging to this system.

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CHEMOTAXIS OF PHAGOCYTES *

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Chemotaxis has been defined as a reaction by which the direction of locomotion is determined by chemical substances in the environment (1). Chemotactic attraction of leucocytes has first been described by Leber in 1888 and his findings were immediately integrated into Metchnikoff's contemporary concept of inflammation. It was postulated that chemotaxis directs mobile phagocytes to the site of injury and thereby promotes efficient phagocytosis and histiolysis (2). For a long time the experimental support for this hypothesis remained rather poor (3). More recent data, however, which have been obtained with a new and more efficient in vitro technique developed by Boyden (4) show a fairly good correlation between *in vitro* chemotaxis and leucocyte accumulation *in vivo*.

It has been shown *in vitro* that a chemotactic gradient results in attraction (positive gradient) and in chemotactic trapping of cells (negative gradient; Fig. 1). As a consequence, leucocyte accumulation is observed in the area with the highest concentration of cytotaxins (chemotactic mediators). In absence of a gradient or in a high cytotaxin concentration, enhanced random migration is observed (5, 6). These *in vitro* findings are in line

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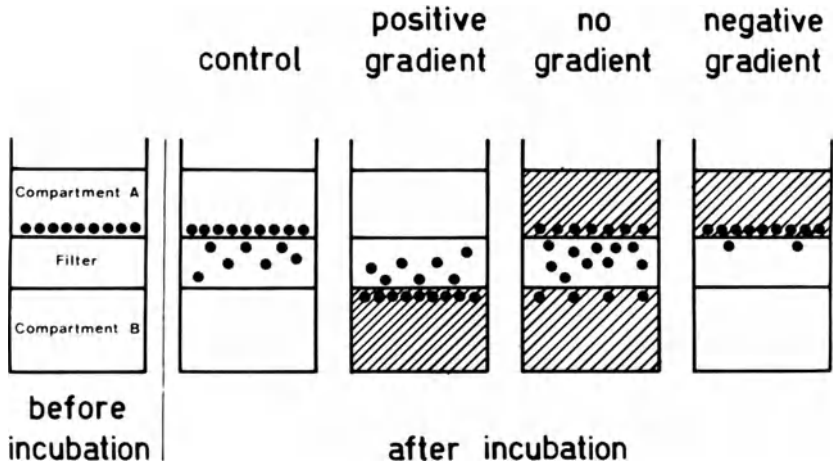

Fig.1: The influence of cytotaxins on leucocyte locomotion in vitro (shaded areas = cytotaxin containing medium). Cells are attracted by a chemotactic solution (positive gradient) or trapped in it (negative gradient), but in absence of a gradient enhanced random migration is observed (no gradient) (5, 6).

with in vivo observations by Buckley (7), who noted directional migration in marginal areas of the inflammatory site, where a gradient is most likely to occur, and random migration in the center, where the cytotaxin concentration is presumably high and relatively uniform. The varying cellular composition of the inflammatory exudate can be explained to some extent by cell specific chemotaxis (6, 8). These data provide a reasonable basis for leucocyte accumulation in vivo by chemotaxis. This concept is supported by the findings that cytotaxins are formed in vivo and that the chemotactic activity of various agents in vitro correlates with their leucotactic activity in vivo (for review see 9). Cytotaxins of different structure and cell specificity are released by bacteria or tissue cells while others are formed in serum or plasma (9, 10). They are all assumed to play their part in directing phagocytes to the site of injury. The following experiments have been set up in order to analyse some other aspects of the relationship between chemotaxis and phagocytosis.

INHIBITION OF CHEMOTAXIS FOLLOWING PHAGOCYTOSIS

Rabbit neutrophils from casein induced exudates (1.9 x 10^6cells/ml) were incubated for 40 minutes at 37°C in either 10% normal rabbit serum alone (NRS), or 10% normal rabbit serum containing 100 latex particles/cell, or in 2% human serum albumin (HSA) in Gey's solution containing 100 latex particles/cell. After washing the cells, the degree of phagocytosis was assessed microscopically and the chemotactic responsiveness of the neutrophils to 10% antigen-antibody treated rabbit serum was determined using Boyden's technique (4, 11). Following incubation in 10% NRS containing latex particles, 90-95% of the cells had engulfed particles and their chemotactic response was significantly impaired (Table 1). If the neutrophils were incubated with latex in 2% HSA in Gey's solution, uptake was small (2-5% of the cells containing particles) and their chemotactic responsiveness was near that of the controls.

cells incubated in	cells/field Exp.1	Exp.2
2 % human serum albumin + latex*	498	191
10 % rabbit serum + latex*	191	8
10 % normal rabbit serum	425	158

*100 latex particles/neutrophil

Table 1: Effect of phagocytosis on neutrophil chemotaxis.

Cell death as assessed by total cell counts and trypan blue exclusion test was below 20% and showed no significant difference between the three groups. In earlier experiments inhibition of neutrophil chemotaxis was observed following phagocytosis of antigen-antibody precipitates (12). This inhibition was, however, accompanied by

an increased death rate in the antigen-antibody treated cells.

It appears that phagocytosis affects amoeboid motion in general (13, 14). The mechanism by which the inhibition is achieved is not clear. Energy necessary for phagocytosis (15) is possibly diverted from motility. Williams and Walters (16) have demonstrated that also in vivo circulating neutrophils fail to emigrate in pleural exudates after phagocytosis of particles such as bacteria or carbon black. Even though up to 90% of the circulating neutrophils had been labelled with particles, the cell count in the exudates was comparable to that of the controls, indicating that migration inhibition of labelled cells is compensated as long as there is sufficient supply of unlabelled cells. Another possible consequence is that cells which have migrated into the inflammatory site and are phagocytosing are thereby temporarily trapped.

RELEASE OF CHEMOTACTIC FACTORS FOLLOWING PHAGOCYTOSIS

Rabbit neutrophils from casein induced peritoneal exudates release some chemotactic activity for neutrophils and macrophages. The release is significantly enhanced following phagocytosis of antigen-antibody complexes. The factors released include cytotaxins (substances with a direct chemotactic effect on cells) as well as cytotaxigens (substances which induce formation of cytotaxins). Leakage of small to moderate activity from cells not treated with immune complexes possibly reflects that these cells have already been stimulated by casein (Table II). For Phelps, studying the formation of intracellular neutrophil cytotaxins, finds that unstimulated human blood granulocytes display no chemotactic activity (16).

Attempts were made to associate the chemotactic activity released from leucocytes with defined subcellular fractions. Rabbit neutrophils from glycogen induced exudates were disrupted and two distinct fractions were obtained by differential centrifugation: a granule fraction and a postgranular supernatant fraction. The cytotaxigens which induce formation of neutrophil cytotaxins in fresh serum are predominantly associated with the granular fraction, whereas the cytotaxins themselves are predominantly found in the postgranular fraction (17). Furthermore, incubation of the granule fraction in plasma or the incubation of the postgranular fraction in serum leads to the formation of macrophage cytotaxins (18). Thus, phago-

Agents tested in Eagle's medium	Neutrophils/ field		Macrophages/ field	
	No NRS	10% NRS	No NRS	10% NRS
Control solutions:				
medium alone	0	36	2	64
medium + immune complex	1	311	1	35
Culture supernates:				
from neutrophils alone	34	153	42	131
from neutrophils with complex	250	339	88	138

Table II: 10 x 10^6 casein induced rabbit peritoneal exudate cells/ml medium were incubated with or without 0.1 mg HSA-anti HSA complexes/ml cell suspension for 2 hours at 37°C. The mixture was then centrifuged and the supernatant tested for chemotactic activity (18).

cytes which have been attracted *in vivo* by chemotaxis may, as a result of their phagocytic activity, release various chemotactic factors capable to attract more phagocytes. This may be reflected in the findings of Spector and Willoughby (19) who found two separate stages of leucocyte emigration in the course of the inflammatory response. The second stage is possibly the result of the chemotactic factors released by the phagocytes which have arrived in the first phase.

Thus chemotaxis and phagocytosis in motile phagocytes are closely linked processes: 1) chemotaxis can induce accumulation of motile phagocytes, 2) phagocytosis inhibits the chemotactic response of these cells, and 3) phagocytosis induces the release of chemotactic substances from such cells.

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CHEMOTAXIS OF PHAGOCYTIC CELLS TOWARDS PROTEINS: THE EFFECT OF PROTEIN DENATURATION[1]

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The phenomenon of chemotaxis has been recognized to be of fundamental biological importance since the observations of Pfeffer in 1884 (1) of chemically-mediated migration of plant sperm and of Leber in 1888 (2) and of Metchnikoff in 1893 (3) on the chemotactic migration of phagocytic leucocytes *in vivo*. However, it was not until 1962 that the first reliable quantitative *in vitro* technique for measuring chemotaxis was described by Boyden (4). He placed neutrophil leucocytes in the upper compartment of a chamber separated from a lower compartment by a filter of a suitable pore size through which the cells could migrate actively but not drop passively. If chemotactic substances were placed in solution in the lower compartment of this chamber, the neutrophils migrated towards them. Several substances have since been shown to be chemotactic for neutrophils, among them the activated peptides C3a and C5a split from complement during fixation by immune complexes or other activators (5-8), substances present in bacterial culture filtrates (9,10) and the soluble fraction derived from homogenized granulocytes or liver cells (11). Macrophage-specific chemotactic factors have also been shown to exist (12,13). Certain substances, e.g. casein,are chemotactic for both types of cell.

It is very likely that the *in vitro* phenomenon of chemotaxis forms a model for the migration of phagocytic cells *in vivo* in the acute inflammatory response and in granuloma formation. Many

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of the substances described above are known to be present in inflammatory lesions and are probably capable of exerting a short-range attraction on cells migrating out of the blood-stream especially in conditions where vascular permeability is increased. It would also be expected that, in such lesions, many of the body's own native proteins might become damaged by enzymatic splitting or other mechanisms and require removal by phagocytic cells. In this paper, the possibility that native proteins become chemotactic for neutrophil leucocytes upon denaturation is explored and a mechanism, based on the results obtained, is proposed by which cells may recognize and migrate towards chemotactically active proteins.

EXPERIMENTAL

Chemotaxis

The chamber used in chemotaxis tests was a simplified and smaller version of Boyden's original chamber (see Fig. 1). The lower compartment was a 5 ml glass beaker. This was filled with 4 ml of the chemotactic substance under test. In this fluid was suspended the sawn-off barrel of a tuberculin syringe to the lower end of which had been glued a circular filter. The cell suspension was placed in the upper compartment before immersion of the latter in the lower compartment. Each test was done in triplicate so that three upper compartments were suspended in each lower compartment. Neutrophils were obtained from heparinized human blood by dextran sedimentation. Macrophages were obtained from the peritoneal cavity

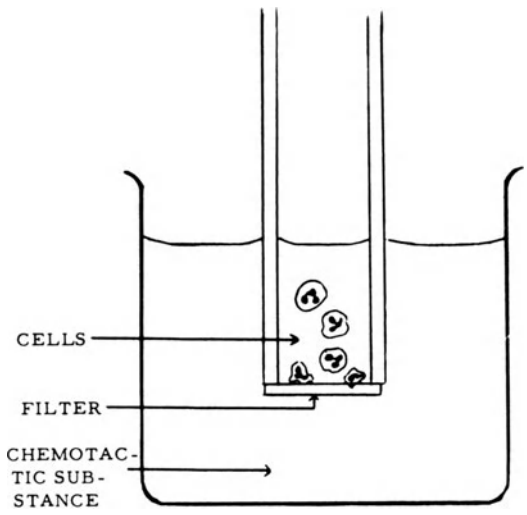

Figure 1. Diagram of the chemotaxis chamber

of the guinea pig 4 days after an intraperitoneal injection of paraffin oil. The washed cells were suspended in the upper chamber in a volume of 0.2 ml at a concentration of 6-8 x 10^6 per ml. The filter used for tests on neutrophils was a 3μ cellulose ester filter (Millipore Filter Corp., Boston, Mass). For macrophages an 8μ filter (Sartorius A.G., Gottingen, Germany) was used. The test substance in the lower chamber was dissolved in Gey's solution containing 100 U. per ml of penicillin and 50 μg per ml of streptomycin. The tests were incubated for 3 hours (neutrophils) or 5 hours (macrophages) at 37° and the filters were then fixed, detached from the syringe barrels, stained with haematoxylin and reversed onto a microscope slide. Neutrophil leucocytes migrate through the thickness of the filter and adhere to the lower surface. They do not drop off, so the count of neutrophils on the lower surface of the filter gives a measure of the migration of cells through the filter.

Substances Tested for Chemotactic Activity

Included in all tests were a negative control: Gey's solution, the medium used for the tests, which is not chemotactic, was placed in the lower compartment of one chamber. The positive control was a solution of crude casein (Merck A. G., Darmstadt, Germany, "Casein nach Hammarsten" or "Casein alkaliloslich") at 5 to 10 mg per ml in Gey's solution placed in the lower compartment of the second chamber. Casein consistently attracts neutrophil leucocytes so that counts of several hundred cells per high power field are recorded on the lower surface of the filter.

The protein used in the majority of experiments was human serum albumin (HSA). This was tested, usually at between 1 and 2 mg per ml, as a chemotactic substance after being treated in the following ways:

a) by heating to 70°C for 10 minutes,
b) by acidification with 1N HCl to pH 2 or pH 4 followed by return to pH 7.2 prior to testing,
c) by foaming. This was achieved by shaking the solution of HSA vigorously for 2 hours in a flask shaker so that most of the liquid present was converted into a foam,
d) by reduction-alkylation, treating the HSA with 0.1M 2-mercaptoethanol followed by dialysis against 0.02M iodoacetamide. The HSA was then returned to physiological conditions, prolonged dialysis being necessary completely to remove iodoacetamide,
e) by treatment with 6M guanidine hydrochloride followed by reduction-alkylation and return to physiological conditions.
f) a control solution of native HSA was tested without any of the above treatments.

All of the above samples were tested for chemotactic activity in Gey's solution at concentrations of between 1-3 mg per ml. As some polymerization occurred during many of the treatments, the samples were passed through Sephadex G100 columns, to separate polymeric from monomeric HSA. The HSA polymer and monomer were tested separately for chemotactic activity. They were also tested for physical evidence that denaturation had occurred. This was done in two ways: a) by viscometry. As a globular protein becomes denatured, its molecular conformation changes from a globular to a linear form. The incidence of molecular collisions increases as the protein assumes a more linear form and hence the viscosity of the solution increases in parallel.

Viscosity was measured in an Ostwald viscometer at 25°C by observing with a stopwatch the time (T seconds) taken for the liquid surface of the test solution to move from one point to another. The ratio of specific viscosity of each denatured HSA solution to that of native HSA was calculated as follows:

$$\frac{\text{Specific viscosity of sample}}{\text{Specific viscosity of native HSA}} = \frac{\text{T sample - T water}}{\text{T native HSA - T water}}$$

b. by measurement of surface activity. A protein is said to be surface active if a surface layer of a solution at equilibrium contains more protein than a layer of the same thickness in the bulk of the solution. Surface activity is likely to be increased by any procedure which exposes hydrophobic groups normally hidden within the molecule. Such hydrophobic groupings will be attracted to a surface, so that the surface concentration of proteins denatured in this way is greater than that of native molecules.

Surface concentration was estimated by Mr. I. C. McKay by measurement of the surface tension of the solution using a torsion balance. A clean, dry coverslip was suspended from the torsion balance into a shallow puddle of the test solution. The force required to remove the coverslip from the surface of the solution was then determined. Full details of this method and of the calculations to derive surface concentration from it are described by Wilkinson and McKay (14). Surface activity was expressed in the form of an index $\Delta\gamma/C$, where $\Delta\gamma$ is the lowering of surface tension due to protein in the solution and C is the protein concentration.

The Chemotactic Activity of Native and Denatured HSA for Neutrophils

The results of representative experiments on the chemotactic activity for human neutrophil leucocytes of HSA before and after

denaturation are shown in Table 1. Native HSA was not chemotactic. The chemotactic activity of denatured HSA varied to some extent with the method used for denaturation. Thus, heat-denaturation rarely resulted in the acquisition of chemotactic activity by preparations of HSA either in polymeric or monomeric form. On the other hand, acid denaturation and reduction-alkylation usually caused HSA to acquire moderate chemotactic activity. After fractionation on Sephadex G100 this activity was usually associated with the monomer peak and polymerized HSA was less active. Other methods of denaturation were less successful. Thus, most preparations of HSA denatured by foaming did not acquire chemotactic activity although occasional preparations did so. After treatment with guanidine-hydrochloride and mercaptoethanol, preparations of HSA became aggregated on return to physiological conditions. No monomeric HSA was left in the preparation and the polymerized fraction showed only a very moderate activity.

TABLE 1: Chemotactic Activity of Polymeric and Monomeric Fractions of Native and Denatured HSA for Neutrophils

Substance under test	Neutrophils/H.P. field (Mean for 3 filters)	
Controls		
Negative: Gey's solution	7	
Positive: Casein 1%	316	
	Polymer fraction ex G100	Monomer fraction ex G100
Native HSA 1 mg per ml	No polymer	9
Heated HSA (70° 10 min) 1 mg per ml	18	17
Acidified HSA (pH 2) 1 mg per ml	17	83
Reduced-alkylated HSA 1.8 mg per ml	17	96
HSA treated with iodoacetamide alone 3.5 mg per ml	No polymer	27

When the chemotactic activity of these preparations of denatured HSA was correlated with changes in their viscosity and with changes in their surface activity, it was found (Table 2) that if evidence of conformational change was present, i.e., if the viscosity and surface activity had increased above those of native HSA, chemotactic activity increased in parallel so that in general, the greater the physical change in the protein molecule, the more chemotactic it became.

TABLE 2: Chemotactic Activity, Viscosity and Surface Activity of Monomer Fractions of Native and Denatured HSA

Substance under test	Neutrophils per H.P. field (mean of 3 chambers)	Viscosity ratio $\frac{\eta sp \text{ test sample}}{\eta sp \text{ native HSA}}$ (2 mg per ml)	Surface activity index $\Delta \gamma / C$ (dyne cm^2/mg)
Native HSA	5	1.00	1.1
Heated HSA (70°, 10min)	1	1.35	3.2
Acid-treated HSA (pH 2)	81	1.61	5.2
Reduced-alkylated HSA	96	2.20	7.9
Reduced-alkylated and heated HSA	85	1.60	5.9
HSA treated with iodoacetamide alone	26	0.90	2.6

The Chemotactic Activity of Native and Denatured HSA for Macrophages

Table 3 shows the chemotactic activity of native and denatured HSA preparations for guinea pig peritoneal exudate macrophages. The results were similar to those obtained with neutrophils. Thus, native HSA was not active. Acid-treated and

reduced-alkylated HSA were both moderately active, although in this case, unlike the neutrophil tests, polymer preparations had an activity which was as great as that of the monomer preparations at the same concentration.

TABLE 3: Migration of Guinea Pig Peritoneal Exudate Macrophages Towards Native and Denatured HSA

Substance under test	Macrophages/H.P. field (mean of 3 counts)	
Negative control Gey's solution	0	
Positive control Casein 10 mg/ml	30	
	G100 polymer	G100 monomer
Native HSA 1 mg/ml	No polymer	4
Reduced-alkylated HSA 1 mg/ml	17	22
Acidified HSA (pH 2) 1 mg/ml	24	16

The Chemotactic Activity of Indian Ink

Carbon particles or suspensions of these particles in the form of Indian Ink are frequently used in tests of reticulo-endothelial function. In this experiment, Indian ink and its constituents were tested as chemotactic substances. Whole Indian ink (Pelikan Carbon Black Dispersion C11/1431a, Gunther Wagner, Hanover, Gemmany) was tested together with its individual constituents which are as follows: carbon particles (size 200-500Å, 10 per cent), fish glue, 4.3 per cent and preservative in water. This ink is diluted for biological use in a 1 per cent gelatin solution. The chemotactic behaviour of these components is shown in Table 4. Fish glue was found to be the active constituent of the ink and carbon particles were inert. Fish glue is, of course,

a mixture of many substances including a considerable quantity of protein in a denatured state.

TABLE 4: The Chemotactic Activity of Indian Ink and its Constituents for Neutrophils

Substance under test	Neutrophils/high power field
Gey's solution	2
Casein 10 mg per ml in Gey's solution	207
Gelatin 10 mg per ml in Gey's solution	3
Indian ink 'Pelikan' 1 drop per ml in 1% gelatin-Gey's	40
Carbon particles (16 mg per ml in 1% gelatin-Gey's)	1
Fish-glue protein ($O.D._{280}$ 2.4) in Gey's	118

DISCUSSION

The experiments on Indian ink illustrate one important difference between chemotaxis and phagocytosis. Although carbon particles are rapidly phagocytosed *in vivo*, they are not chemotactic for motile phagocytes *in vitro*. Chemotaxis is, of necessity, mediated by diffusible, soluble substances which can set up concentration gradients which can be detected by phagocytes at some distance from the source of the substance. Thus, it is soluble fish-glue proteins in Indian ink, probably in a denatured state, rather than the carbon in the ink which induce chemotaxis.

The correlation between conformational change in HSA molecules as measured by viscosity and surface activity and the acquisition of

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chemotactic activity by these molecules would imply that there are groupings on protein molecules which are recognized by cells and which stimulate chemotaxis of those cells. These groupings are concealed in the native state, but when the protein opens out during denaturation, they become exposed. The difference in chemotactic activity of HSA molecules denatured in different ways would support this idea. Heat-denatured HSA becomes polymerized by disulphide bond interchange between molecules (15) probably without significant unfolding of the molecule (16). This method of denaturation does not make it chemotactic. On the other hand, acid denaturation of HSA, the physical chemistry of which has been studied in some detail, leads to unfolding of the molecule so that hydrophobic areas, normally concealed in the interior, become exposed (17). This preparation acquires chemotactic proterties. There is no direct evidence, at present, as to the nature of the chemotactically-active groups on protein molecules. These could be of several types but we have speculated elsewhere (14) that hydrophobic groups are of importance in chemotaxis. These groups

are, to a large extent, concealed on native proteins and may be exposed during denaturation (see Fig. 2). Exposure of such groups leads to an increase in the overall hydrophobic properties of the protein molecule so that the protein will show an enhanced affinity for interfaces where the hydrophobic groups can position themselves away from the water phase. In other words, such proteins become more surface active. It is possible that surface-active protein molecules may concentrate not only at water-air surfaces but also at the surfaces of cells. Thus, selective concentration of these molecules may trigger enzymatic mechanisms of the types postulated by Becker and Ward (18) as initiators of directional cell movement or, on the hypothesis of Woodin and Wienecke (19), such molecules may act at hydrophobic surfaces on cell membranes, the orientation of which is stated by these authors to control cation exchange in the cell and thus, ultimately, its chemotactic activity. It may be relevant, in this context, that casein which was used as a positive control substance in all of these experiments, normally has a random coil conformation in aqueous solution (20,21), a molecular form which other proteins only acquire after denaturation and which would be likely to prevent the hydrophobic side-chains of the casein molecule from taking up a position in the interior of the molecule as is the case in most other globular proteins.

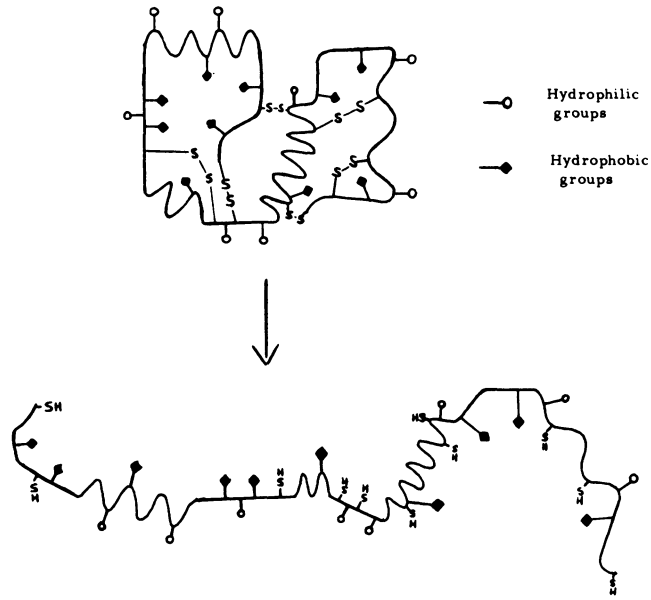

Figure 2. Schematic drawing of a hypothetical single-chain protein molecule in its native state (above) and after splitting of disulphide bonds and unfolding (below) showing how hydrophobic side groups, normally concealed in the interior, may become exposed to the aqueous environment during denaturation.

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LEUCOCIDIN, DFP, THE LEUCOCYTE POTASSIUM PUMP AND THE INHIBITION OF CHEMOTAXIS

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It is a reasonable hypothesis that phagocytosis and motility in the polymorphonuclear leucocyte* depend on special properties of the cell membrane that are absent from membranes of tissue cells. Any clue to the nature of these characteristics may be of value in identifying the mechanism of phagocytosis and motility. I describe here some unique properties of the leucocyte surface that have been revealed by studying the cytotoxic action of leucocidin [1,2]. The evidence that the mode of action of leucocidin is relevant to the mechanism of phagocytosis and motility is circumstantial, depending on the modification of all three phenomena by organophosphorus compounds. Nevertheless, I hope the new data made available will enable a new approach to be made to the mechanism of chemotaxis and phagocytosis.

The nature of leucocidin (3). Leucocidin is an extracellular product of the staphylococcus, consisting of two proteins, that kills the leucocytes and macrophages of rabbit and man. The two proteins, called the F component and the S component of leucocidin, are globular, water soluble, have molecular weights of about 30,000 and have both been crystalised. They act synergistically; neither is toxic alone. This article is concerned with the action on the leucocyte.

The response of the leucocyte to leucocidin (2). The immediate response is an increased permeability to cations. Amino acids, phosphate esters, nucleotides or reducing sugars are retained in the cytoplasm at 6-30 times their concentration in the medium. Accompanying the increased cation permeability is an

*called leucocytes in this article.

accumulation of orthophosphate in the cytoplasm at the expense of acid-soluble phosphorous compounds. Degradation of acid-insoluble material does not occur. Subsequent changes are dependent on extracellular calcium. If it is present, the proteins in the cytoplasmic granules are secreted into the medium, calcium is accumulated in vesicles derived from the empty granules and an orthophosphate-nucleotide exchange reaction is set up on the cell surface. The secretion of the granule proteins occurs by exocytosis[4] (fusion of the cell surface membrane with that of the granule). There is considerable evidence for this mechanism and of particular importance is the way in which the granules move. In the normal cell they can be seen under the light microscope to be moving in streams, but if leucocidin is added, they move in a random fashion. N-ethyl maleimide (1 mM) added to leucocidin-treated cells in the absence of calcium stops the granules and, immobilised in the cytoplasm, they cannot make contact with the cell membrane[5]. Subsequent addition of calcium does not induce secretion.

If calcium is added to leucocidin-treated cells prepared in its absence (and in the absence of N-ethyl maleimide), secretion of the granule proteins and the other calcium-dependent reactions are induced. It is not necessary for calcium to be present at the same time as leucocidin for the calcium-dependent reactions to occur[6]. This finding is of great importance in the analysis of the response to leucocidin for it is known that leucocidin acts on the outside of the cell membrane (see below) and it can be neutralised at any time by adding antibody. It has been found that the calcium-dependent reactions can be induced in the leucocidin-treated cell after the excess leucocidin has been neutralised with antibody. The cell has the necessary reagents once contact of the granules and the membrane has occured. By adding reagents before or after leucocidin has been neutralised with antibody, it is possible to determine if they modify the action of leucocidin or the response of the cell. This is of value in identifying the site of action of drugs on the cell (see below).

The site of action of leucocidin. This is known unambiguously to be the outside of the cell surface for if leucocidin labelled with I^{131} is added to an excess of cells, it is not adsorbed to any great extent but it is all inactivated[7]. This inactivation is synergistic between the two components of leucocidin and the mass of leucocidin that can be inactivated by cells is equal to the mass of leucocidin required by produce a maximum cytotoxic effect. This is about 200,000 molecules/cell and occurs when the leucocidin concentration is 10^{-7}-10^{-8} M. The synergistic inactivation of leucocidin is also brought about by cell homogenates, providing a method to identify cell membrane fractions in cell homogenates and to control the purification of the cell membrane by differential centrifugation.

Now if membranes are treated with phospholipase A (which hydrolyses esterified fatty acids from phospholipids) the membranes no longer inactivate leucocidin. At physiological ionic strength, the only phospholipid to mimic the inactivation of leucocidin by cells and membranes is triphosphoinositide, suggesting that it may be present at the site of action of leucocidin[8]. At low ionic strength, the F component interacts with numerous phospholipids, and following its interaction it can form polymers with the S-component of leucocidin. The significance of the interaction of phospholipids with leucocidin at low ionic strength to the mode of action of leucocidin has been discussed elsewhere[9]. The relevance here is that the nature of the phospholipids that interact indicates that the esterified fatty acids are of importance, as they are in the interaction of leucocidin with the membrane.

The action of leucocidin on isolated membranes. Leucocidin does not solubilise or degrade membrane components, change the light scattering properties or alter the intensity or flourescence of the dye 8-anilino naphthalene-1-sulphonate adsorbed on the membrane. The activity of the ATPase, β-glycerophosphatase or adenosine monophosphatase is not affected by leucocidin. The acyl phosphatase of the membrane with p-nitrophenol phosphate or acetyl phosphate as stimulated 2-fold by leucocidin. This stimulation is synergistic between the two components of leucocidin, it occurs with masses of leucocidin and membrane that produce maximum inactivation of leucocidin and is not observed with other toxins[1]. If membranes are treated with phospholipase A, leucocidin fails to stimulate the acyl phosphatase and phospholipase A can reverse the stimulation induced by leucocidin in normal membranes. Treatment with proteolytic enzymes is without effect in both cases. It has also been found that the acyl phosphatase solubilised by detergents is not stimulated by leucocidin. It is clear that esterified fatty acids of phospholipids are essential for stimulation of the membrane phosphatase[10].

Properties of the acyl phosphatase of the leucocyte membrane[1,10]. The activity is maximal at pH 5 but smaller peaks are found at pH 6.2 and pH 7.2. The overall activity and the position of the peak activities is changed by treating the membranes with dilute tris buffer or with concentrated sodium iodide solution and these reagents induce a sensitivity to potassium over the range pH 6-8. This stimulation is prevented by high concentrations (10^{-4}M) of ouabain. Leucocidin stimulates over the range pH 4.5-8. The stimulation is additive to that of potassium and is not inhibited by ouabain. It has been argued that the multiple pH optima may result from conformational changes in the membrane rather than the presence of more than one enzyme[1]. Trypsin, but not phospholipase A, prevents the potassium stimulation - the opposite is found with leucocidin stimulation. Phospholi-

pase A changes the activity in different ways according to the pH at which it is measured. At pH 7.2 the activity of phospholipase A-treated membranes is smaller than that of the controls while at pH 6.2 or at 7.2 the activity is greater and approaches that of leucocidin-treated membranes. The enhanced activity of phospholipase A-treated membranes is reversed if acidic phospholipids are added. Similarly, if acidic phospholipids are added to the solubilised acyl phosphatase, the activity is suppressed. The activity of leucocidin-treated membranes is inhibited by phospholipids but this also happens with normal membranes and the percentage stimulation produced by leucocidin is retained in the presence of added phospholipids. Similarly, phospholipids do not induce a sensitivity to leucocidin in phospholipase A-treated membranes or solubilised acyl phosphatase. Thus, although the stimulation of the acyl phosphatase by leucocidin is mediated through an interaction with the esterified fatty acids of phospholipids, the effect is highly specific suggesting that the relevant phospholipids are attached to the membrane in a novel way.

The unique character and the function of the acyl phosphatase. Acyl phosphatase activity has been observed in membranes from brain [11] and electric organ[12]. The enzyme in these tissue cells is quite distinct from that in the leucocyte. The stimulation by potassium is much greater, it is confined to the activity at neutral pH, it is inhibited by low concentrations of ouabain, and it is dependent on the presence of phospholipids. With solubilised acyl phosphatase from tissue cells, phospholipids stimulate the potassium-sensitive activity. In all these respects the leucocyte enzyme behaves in a different way. In tissue cells there is evidence that the acyl phosphatase is part of a sodium sensitive-ATPase. The leucocyte membrane does not possess a sodium-sensitive ATPase[1].

The mechanism of electrolyte control in the leucocyte correlates with the pecularities of the membrane phosphatases. If leucocytes are cooled to 0°, the potassium content falls and the sodium content rises. On warming the cells to 37°, the potassium concentration is restored to about its original level, but the sodium concentration is only partly reduced and the cells remain swollen[13]. In contrast to most mammalian cells, the leucocyte appears to regulate its electrolyte balance by controlling the potassium concentration alone. For these reasons it has been concluded that the potassium-sensitive acyl phosphatase is part of an electrogenic pump specific to potassium[1].

On this basis, the permeability change induced by leucocidin can be regarded as a result of a structural change in the potassium pump of the cell.

The action of drugs on the leucocidin-treated leucocyte. It appeared probable that the acyl phosphatase would be only part of the potassium pump in the cell. Indeed it is clear that phospholipids are closely associated with the acyl phosphatase in the membrane. It is possible that other structures and reactions are coupled with the enzyme activity and with the mode of action of leucocidin. I therefore sought for drugs that specifically modify leucocidin action. These can be identified by the device mentioned earlier, that enables reagents that modify leucocidin action to be distinguished from those that modify the response of the cell. Drugs that affect leucocidin action should alter all the responses of the cell only if they are added before leucocidin has been neutralised by antibody. They should have no effect on the responses of the cell if they are added after leucocidin has been neutralised. They should also have no action on the cytotoxic action of streptolysin O or excess Vitamin A.

Tetraethylammonium ions inhibit the action of leucocidin [1] and organophosphates such as di-isopropylphosphoflouridate (DFP) enhance the action of leucocidin[14]. Neither reagent affects the activity of the membrane acyl phosphatase and it can be concluded that they modify the potassium pump in a different way from leucocidin. Tetraethylammonium ions have no action on the normal cell and it is possible that they block the ion-pathway activated by leucocidin and that it is only in the presence of leucocidin that receptors become accessible. This is analaogous to the action of tetraethylammonium ions on the axon. DFP has the opposite action to TEA suggesting that it stabilises the ion-pathway activated by leucocidin.

There is independent evidence in support of this. The dose-response curve shows a plateau of no cellular response to low leucocidin concentrations, indicating that the cell can reverse the action of sub-optimal amounts of leucocidin. The conversion of leucocidin to an inactive form prevents multiple collisions from mimicking the effect of higher concentrations. DFP only enhances sub-optimal amounts of leucocidin. It has no effect when leucocidin is in excess. When DFP is present the dose-response curve of leucocidin action does not show the plateau of no response to low leucocidin concentrations. DFP does not prevent the cell from inactivating leucocidin and it appears that DFP stabilises the ion-channel activated by leucocidin. Furthermore, the demonstration that on the leucocidin-treated leucocyte DFP acts as a detergent is consistent with its stabilising hydrophobic surfaces separated by leucocidin[15].

The physiological significance of the mode of action of leucocidin and DFP. The reversal of the action of sub-optimal amounts of leucocidin suggests that the separation of the surfaces with which leucocidin interacts may also occur under

physiological conditions. In support of this, DFP can inhibit the reaccumulation of potassium in depleted cells and even induce a leakage of potassium from normal cells. The sites with which DFP interacts are present in normal cells. Now DFP and other organophosphates inhibit phagocytosis and chemotaxis under the same conditions as they enhance leucocidin and inhibit potassium reaccumulation. It is possible that the integrity of the surfaces in the membrane with which leucocidin and DFP interact is essential for phagocytosis and chemotaxis.

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HYPOTHESIS: A POSSIBLE RELATIONSHIP BETWEEN BLOOD CLOTTING AND THE RECOGNITION BY THE BODY OF FOREIGN PARTICLES

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INTRODUCTION

The ability of cells to recognize other cells or particles is a general and fundamental problem in biology. It is basic to the survival of protozoa which must differentiate food from non-food and equally basic to the multicellular animal whose integrity depends upon the ability of its component cells to recognize and respond to cells of a similar or dissimilar type (1,2). Almost without exception when foreign particles enter the mammalian blood stream they are recognized and ultimately ingested by phagocytic cells. This is true whether the particles are parasites which enter the body accidentally, or vaccines or test materials which are introduced deliberately. The ability to distinguish aged, damaged or effete isologous cells from normal cells and the ability to recognize, or not, normal as opposed to neoplastic cells is undoubtedly part of the same phenomenon.

While a great deal of work has been done on phagocytosis which has been the subject of several reviews (3,4,5,6), and on the clearance of foreign material from the blood stream by the reticuloendothelial system (RES) (7), surprisingly little work has been specifically directed to the problem of what is responsible for "recognition" by the host. Some investigations have been made of the surface chemistry of various cells and synthetic model particles, on the assumption that the contact between phagocyte and particle is analogous to the particle-particle interaction considered in colloid stability theory. Colloidal systems become unstable if the energy of repulsion between the particles, which is predominantly electrostatic, is reduced. This has given rise to

several studies of the electrokinetic or zeta potential measurements of cells or test particles (8). Although many interesting results have been obtained, they are generally disappointing in that they have shown only that surface potential alone is not enough to explain recognition. Bangham et al (9) found that the polymorphonuclear leucocyte, lymphocyte, platelet and erythrocyte have very similar electrophoretic mobilities in saline and on the basis of charge alone should remain dispersed, i.e. phagocytosis of erythrocytes should never occur. Wilkins and Bangham (10) could not explain the phagocytosis of polystyrene latex or starch grains on the basis of the surface charges of the cells and the particles. This has led to the suggestion that proteins or other macromolecules may be necessary for cell-cell interaction. Perkins and Leonard (11) found an increase in phagocytosis as a function of the phylogenetic distance between a phagocytic cell and red cells from various species, in spite of the fact that the differences between surface potentials and red cells from different animals is very small (12). Finally although young isologous red cells circulate unhindered and old red cells are readily removed from the circulation, it was shown (13) that only very small differences exist in the electrophoretic properties of young and old red cells. One of the most impressive features of phagocytosis in fact is the fantastic variety of particles which are recognized apparently independently of surface charge. One can generalize in fact that it is only healthy isologous cells which escape detection. This difficulty has led to the search for a common pathway and many attempts have been made to find a recognition factor or recognition factors in the blood.

Mudd et al (3),in a study of phagocytosis of bacteria found good agreement between agglutination, electrophoretic mobility and other surface properties, and phagocytosis. All of these properties in turn could be related to deposition of serum protein on the bacterial surface and they pointed out the importance of this in terms of protein being able to modify very dissimilar parasite surfaces and render them similar with respect to the surface properties upon which phagocytosis depends.

There seems little doubt that the opsonins first described by Wright and Douglas (14) are natural antibodies contained in blood. Several other factors such as the "phagocytosis promoting factor" (15) have been isolated having a broad activity. Recent work by Jenkins and Rowley (16) and Saba and Di Luzio (17) has established the importance of opsonins and showed that so called RES blockade is simply a temporary loss of opsonins from the circulation which can be restored with fresh serum or prevented from reforming by previous treatment with puromycin. In short everything points to the necessity of proteins for recognition.

During the course of experiments on the clearance of colloidal particles in heparinized animals (18,19),it became clear that one could make out a case for a relationship between blood coagulation and recognition. A study of the literature has given support to the following hypothesis:

1. Foreign particles with a wide variety of surfaces are recognized and dealt with by the RES.
2. Almost all foreign surfaces which have been investigated initiate blood coagulation.
3. Intravenous fibrin is cleared by the RES.
4. It is therefore suggested that foreign particles, upon entering the circulation,become coated with a layer of fibrin. It is then this fibrin layer which is recognized and the fibrin coated particle which is phagocytosed by the RES cells.

Evidence from the literature in support of this hypothesis will be presented under four headings:

EVIDENCE FROM EXPERIMENTS ON PHAGOCYTOSIS OR INTRAVASCULAR CLEARANCE OF PARTICULATE MATERIAL

Probably the first suggestion of fibrin involvement in recognition came from Knisely et al (20). By directly observing living tissue in as non-traumatized a manner as possible it was observed that most particles injected into the blood stream became coated as a prelude to phagocytosis. The coating consisted of a "refractile fibrin-like material" which was not found on native red cells or undamaged endothelium. It was not found,furthermore,if the animal was previously heparinized. It was not possible,of course,to positively identify fibrin but similar evidence for the same idea was found using the rabbit ear chamber (21). Upon injection of bacteria,"platelets appeared to hang together as if on invisible threads, probably fibrin". Thomas (22) found that an injection of endotoxin produces a fibrin-like coating on the endothelium which may be prevented by previously heparinizing the animal.

There is now a considerable body of work on indirect measurements of phagocytosis <u>in vivo</u>, which concerns itself with the rate of removal of foreign particles from the blood stream. Such particles accumulate in the spleen, liver, lymph nodes and bone marrow (see reviews by Halpern (23) and Howard (5)). Biozzi et al (7) in a series of papers showed that under properly chosen conditions, "inert" colloidal particles or bacteria are cleared

at an exponential rate, the slope of the clearance depending upon the injected dose. With larger doses, however, considerable accumulation occurred in the lungs. It seems reasonable to postulate the following explanation for this. At low concentration, particles become coated with fibrin but the dilution is such that they remain as single particles and are taken up by the liver and spleen. At high concentrations, particles can agglutinate and form a thrombus of several particles in a fibrin network which is trapped in the narrow capillaries of the lungs (24). Such lung accumulations are gradually resolved and the particles later found in the liver or spleen. Halpern et al (25) also found that the accumulation in the lungs of high doses of carbon could be prevented by using previously heparinized animals. Wilkins and Myers (8), in a study of the effect of altering the surface chemistry of synthetic colloids on their interaction with the RES, found that positively charged colloids also accumulate in the lungs. This also was presumably due to the formation of thrombi since no lung accumulation was shown by heparinized animals (18). The action of heparin, however, must be treated with some caution since it has a broad spectrum of action. Thus, although MacIntyre et al (26) found that it inhibits the clearance of colloidal gold, Filkins et al (27) found that in some *in vitro* systems phagocytosis is considerably increased in the presence of heparin. Another type of experiment which strongly supports the general hypothesis is the observed decrease of blood fibrinogen upon giving large doses of colloid intravenously. Thus, Wiedmeier et al (28) found with "blocking" doses of various colloids a depressed fibrinogen level, while with Syton, a commercial silica preparation, they were unable to detect any circulating fibrinogen. Halpern et al (25) also found depressed fibrinogen levels which could be prevented by previous heparinization.

EVIDENCE FROM IN VITRO AND IN VIVO EXPERIMENTS ON BLOOD COAGULATION

The ability of a foreign surface to initiate blood coagulation has been well-known since the time of Freund (29). In practical terms, haemostasis is the end result of the coagulation of blood initiated by contact with damaged endothelium and/or the blood/air interface, both foreign surfaces in this context. Although stationary blood has a greater tendency to clot it will stay liquid for several hours in a healthy blood vessel, whereas damaged endothelial cells were shown to encourage clotting and decrease clotting times in vitro (30). In the present context, it is tempting to speculate that here is an analogy with the way in which for example effete red cells are removed from the

circulation, i.e. an effete cell is one which is damaged or worn out sufficiently so that coagulation is initiated on the surface and by its coating of fibrin it is therefore recognized.

The blood-air interface is presumably "foreign" by virtue of its adsorbed layer of surface denatured proteins, but investigations have revealed that with the exception of undamaged endothelium,all surfaces are "foreign". Based upon the finding that paraffined glass preserves blood in the liquid state for a longer time than clean glass,it has long been reasoned that hydrophobic surfaces are less thrombogenic than hydrophilic ones. While this is to an extent true and for example siliconized glass promotes clotting less than clean glass, it was realized by Lovelock and Porterfield (31) that the endothelium is not hydrophobic. They were able to show,in fact,that sulphonated polystyrene tubes preserve the liquidity of blood better than untreated hydrophobic tubes of the same material. Numerous attempts have been made to understand the mechanism by which a foreign surface initiates clotting, but such studies are complicated by the very fact that all surfaces trigger the reaction. Studies on the adsorption of various components from plasma by streaming potential, fluorescent antibody and elipsometry measurements (32,33) would indicate the preferential adsorption on a surface of beta-globulins or fibrinogen. Bangham (34) and later Papahadjopoulos et al (35) were able to relate the electrokinetic surface charge of lipid emulsions to their effect on clotting times. The charge was varied by adding varying amounts of either dicetyl phosphoric acid or C_{22} pyridimium bromide to lecithin before emulsification,and,in general, the higher the negative charge the shorter the clotting time. This may explain the somewhat paradoxical result of Wilkins and Myers (8) who found that colloids with a negative surface potential were cleared more rapidly than those with a positive surface potential. This is contrary to what would be expected from considerations of theories of colloidal stability. However,if we consider that a negative surface more rapidly initiates coagulation, it is perhaps easier to understand. The attempt to find or design a non-thrombogenic surface capable of being applied to plastic or other materials has been stimulated by the search for materials for implantation in man as artificial organs or parts (36). Most of this research has been, necessarily, empirical and indeed some of the more useful materials are either very hydrophobic or depend upon the binding of heparin at the blood-material interface. It would seem to the author that more consideration might be given to attempting to imitate a healthy endothelial or red cell surface. A very interesting observation was made by Robbins and Stetson (37) who showed that addition of antigen to immune whole blood caused a marked shortening of the coagulation time. Thus,it seems as

though an antigen-antibody complex which furthermore is known to be readily bound by phagocytes (38), is a strong initiator of blood coagulation and thus provides a connection between the present hypothesis and the enhancement of phagocytosis by specific antibodies. It would be interesting to know, in view of the claim that opsonins are natural antibodies (2), whether foreign particle-opsonin complexes also enhance blood coagulation.

EVIDENCE FROM EXPERIMENTS ON THE INTRAVASCULAR CLEARANCE OF FIBRIN

The suggestion is that a foreign surface or perhaps a foreign surface plus complexed antibody initiates coagulation, that is to say some fibrin is deposited on the particle surfaces. Thus, it is important to establish that fibrin is recognized and removed by the RES, and there seems to be abundant evidence for this. To quote Monkhouse and Milojevic (39): "Under normal conditions blood remains fluid within the blood vessels despite the continuous turnover of plasma proteins involved in coagulation. Evidently, a delicate balance exists between the rate at which coagulants are activated and neutralized. It is only when this balance is disturbed, locally or generally, that signs of intravascular clotting occur". These same authors (40) found that infusion of thrombin and/or thromboplastin in dogs or rabbits at high enough doses was capable of completely defibrinating the animal. Lee (4), in an investigation of the Schwartzmann reaction, infused thrombin slowly into rabbits and showed by electron microscopy that the formed fibrin was cleared by the Kuppfer cells of the liver, while Lee and McCluskey (42) induced intravascular clotting with endotoxin and the fibrin was again shown to accumulate in Kuppfer cells and splenic macrophages by electron microscope and fluorescent antibody techniques. Barnhart (43) also found that thrombin induced thrombi were removed by RE cells and further that the exudates from inflamed joints are rich in neutrophils which can be shown by immunofluorescence to contain quantities of fibrin. It is of interest also that Good and Thomas (44) showed that the first of the two endotoxin injections normally required to provoke a generalized Schwartzmann reaction can be replaced by an injection of colloidal thoria and they proposed that this first injection blockaded the RES so that it was unable to clear the introvascular fibrin produced by the endotoxin. Finally, Se^{75} labelled exogenous fibrin was shown to be rapidly cleared by Kuppfer cells in the rat isolated perfused liver (45).

EVIDENCE FROM CLINICAL STUDIES AND MISCELLANEOUS EVIDENCE

In this section some observations will be discussed which have to do with clinical observations of fibrinogen levels. Petermann (46) pointed out that in acute infections the plasma fibrinogen may be increased and Wells (47) states that in extreme cases such as major trauma or very severe burns, the plasma fibrinogen may exceed 1000 mg/100 ml (normal is 160 mg/100 ml). If we assume that fibrinogen is necessary for recognition of foreign material, it seems very reasonable that it should be under some sort of control which could respond in this way. Conley et al (48) found no circulating fibrinogen in a patient with a massive *E. coli* septicemia and it has been suggested (49) that the lack of fibrinogen in very severe infection is due to liver damage. While this is a very reasonable suggestion, some thought should perhaps also be given to the possibility that in a severe infection the fibrinogen is removed by interaction with the bacterial surfaces faster than it is produced, which may or may not be complicated by reduced fibrinogen synthesis in the case of liver damage. It is interesting that endotoxin, which was shown to provoke a "fibrinoid" deposit on the endothelium (22) in the rabbit ear chamber, can also produce diffuse intravascular clotting at appropriate doses. This may perhaps be linked to the finding of widespread intravascular thrombi in the terminal stages of experimental anthrax and pneumococcal septicaemia (50,51).

Very few cases have been reported in the literature of congenital afibrinogenaemia. One would expect, if the hypothesis is correct, that a lack of fibrinogen would make it very difficult for the host to recognize and hence to deal with invading organisms. In fact de Vries et al (52), and Frick and McQuarrie (53) found that patients with congenital afibrinogenaemia succumb to overwhelming infections at least as often as they do to haemorrhage and in fact the latter authors found transfusion with fibrinogen offers protection against infection.

Finally it is well known that calcium ions are essential for blood coagulation and for phagocytosis (54,10) and the optimum level of calcium ion for *in vitro* phagocytosis was found by the latter authors to be the same as the normal physiological blood level. However, the exact level of Ca^{++} required *in vivo* for optimum blood coagulation is not yet known (55).

DISCUSSION AND CONCLUSIONS

Within certain limitations, there seems to be a good case for seriously considering the involvement of the clotting mechanism

in the recognition problem. The hypothesis, so far as it goes, can be fairly well supported by the evidence already in the literature. The story is however obviously far from complete and this attempt was made simply to bring together various apparently unrelated facts in the hope that it will stimulate experiments and ideas in this obviously important area. Any complete account of recognition would have to deal very thoroughly with considerations of specific antibodies and opsonins and for simplicity the present hypothesis completely ignores the many references to the role played by platelets in phagocytosis. Further, although one can explain phagocytosis in vitro in the absence of protein (6,10) simply on the basis of the experimental system ensuring many particle-cell collisions, which it is presumed will inevitably lead to phagocytosis, in the case of a protozoon selecting and ingesting prey in salt solutions, one has to look elsewhere for mechanisms. It would strengthen the hypothesis considerably if the suggestion made by Knisely et al (20) could be proved and it could be shown for example that foreign particles which are cleared, are always coated in vivo with fibrin, and that fibrin was inevitably present in phagosomes. Also, in view of the differences in the rate of removal of virulent and avirulent pairs of the same bacterial strain, (16) it would be interesting if one could show a differential ability in such pairs of organism with relation to initiation of clotting or say fibrinogen binding.

The question of why fibrin is recognized is one which will ultimately have to be dealt with, possibly in terms of the interaction between cell surfaces and particle surfaces with various adsorbed macromolecules such as fibrinogen and fibrin. It is clear however, that we need to know more about the molecular architecture of cell surfaces before such problems can be resolved. Hopefully, the above hypothesis is useful, since while it does not propose a mechanism in terms of molecular biology, at least in the absence of any other hypothesis it proposes a common pathway which is subject to experimental test. Finally, it is important to stress once more that in the consideration of such a complicated problem, certain aspects have had to be ignored and certain simplifying assumptions made. It is to be hoped that such simplifications have not led to an hypothesis which is so naïve as to have only a very restricted usefulness.

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THE STIMULATING OR DEPRESSING EFFECT OF VARIOUS DRUGS ON THE PHAGOCYTIC FUNCTION OF THE RES

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The reticuloendothelial system is involved in a variety of biological processes. Its functions include the clearance of bacteria, fibrin, and foreign particles from the blood stream, the detoxification of toxic substances, the neutralization of enzymes, the phagocytosis and transfer of lipids,the synthesis and hydrolysis of cholesterol, the conjugation of steroids, and the first step of the immune response, the phagocytosis and transfer of antigen.

The functional state of the RES appears to be regulated by the activity of the hypophyseal-adrenal system, since hypophysectomy abolishes the possibility of RE-stimulation, while high cortisone levels inhibit phagocytic and detoxifying activity of the RE-cells. Furthermore, RE-activity is very sensitive to oxygen deficiency which may be the consequence of reduced blood flow and hematologic or pulmonary changes. On the other hand, RE-activity can be blocked or stimulated by a variety of drugs and it is the purpose of this study to prove the effect of substances widely used in medicine. Especially patients before and after surgery demonstrate often the clinical aspects of impaired defense mechanisms and may possibly experience further impairment by certain drugs.

METHOD

Recent investigations demonstrated a close relationship between phagocytosis, antibody formation and detoxification by the RE-cells. In order to obtain objective data on one

parameter of RE-function, the Carbon-Clearance-Test was used, which was introduced in 1953 by Biozzi and Benacerraf (1). This test consists of the intravenous injection of 16 mg/100 g of carbon (India ink No. c11/1431 a from Gunther Wagner, Hannover, Germany) and the determination of its half-life in the blood by means of blood samples taken after 5, 10, 15 and 20 minutes. The content of carbon still in the serum is measured spectrophotometrically.

In the following experiments, usually the maximum daily doses for humans, calculated for rats or mice, were given as a single injection or as a longtime study on 5 consecutive days.

ANTIBIOTICS

Chloramphenicol and Tetracycline are broad-spectrum antibiotics often used prophylactically in severely injured patients, which block the synthesis of proteins also in mammilian cells by preventing the deposition of t-RNA on the ribosomes. It has been known for some years that they impair antibody formation and Actinomycin f.i. is applied as the strongest immunosuppressive drug in rejection crises after kidney transplantation. A single dose of tetracycline or chloramphenicol resulted in a depression of phagocytic activity for about 48 hours, whereas penicillin and gentamycin did not impair phagocytosis - the latter probably as a result of the lower level of bactericidal activity (Fig. 1).

A third degree burn of 30 percent of the body surface is a severe injury in rats and depresses phagocytic activity for 24 hours. Chloramphenicol and tetracycline given at the same time extend for the RE blockade up to 72 hours and increase post-burn mortality from under 10 percent in controls up to 34 or 43 percent. Therefore, the prophylactic use of these two antibiotics should be indicated cautiously, especially since Swedish studies have shown that their application does not lower the incidence of postoperative pneumonia.

PLASMA EXPANDERS

In shock or after severe injury, plasma expanders are a common first aid treatment. Since rats frequently show allergic reactions towards the dextran, mice were injected slowly, intravenously, with quantities of 0.5 ml plasma expander. In mice, dextran 40 (Rheomacrodex) effected only a short RE-depression for about 6 hours, while dextran 60 (Macrodex) and gelatin solution (Haemaccel) suppressed the phagocytic activity of the RES up to 24 hours (Fig. 2).

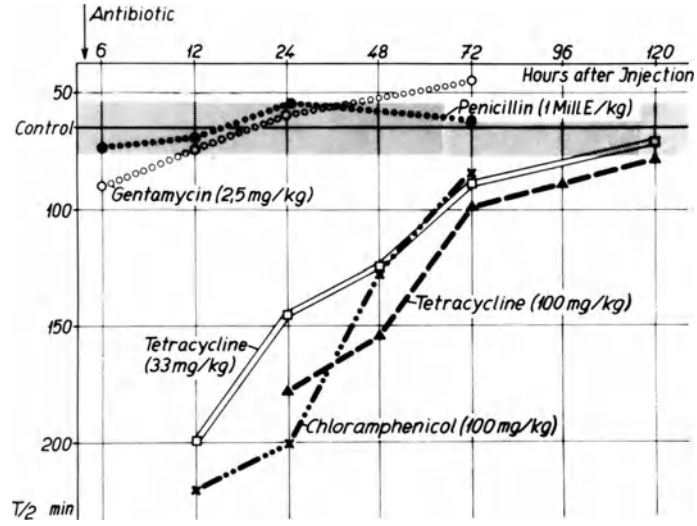

Figure 1: Carbon-Clearance-Test after a single dose of an antibiotic in rats. Tetracycline and Chloramphenicol have a RES-depressing effect for 72 hours.

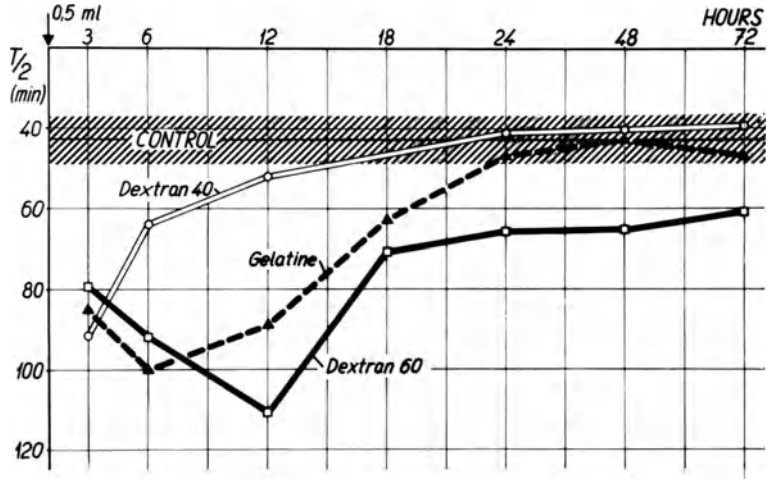

Figure 2: The injection of 0.5 ml equal 1000 ml in a man of dextran 60 or gelatin solution inhibits phagocytosis for 18 hours in mice.

In severely burned mice, dextran 60 and gelatin injections resulted in a slight additional prolongation of the RES-depression. Only the administration of dextran 40 had a beneficial effect on burn shock and on phagocytic activity, which could be compared with that of the saline injected controls (Fig.3). The RE depressing effect of dextran solutions was related to the molecular weight which ranges from 40.000 to 60.000 and 120.000 in solutions used in England. The higher the median molecular weight, the better and longer lasting is the volume expanding effect.

BURN TREATMENT

Cold water therapy is the treatment of choice of a fresh burn. Applied immediately after a scald, it prevents the conversion of a second, into a third degree burn. This leads to a shorter period of shock and consequently to less impairment of RE function.

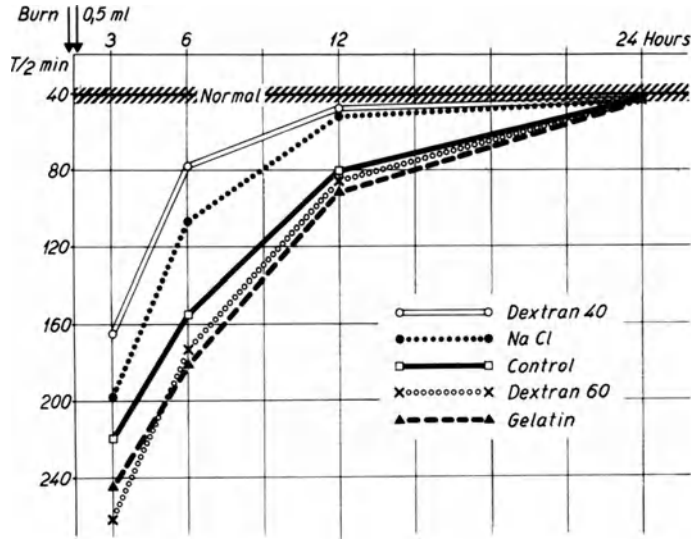

Figure 3: In severely burned mice, dextran 60 and gelatin prolong RES-blockade; only dextran 40 has a beneficial effect.

From three different temperatures, cold water of 20°C proved to be best in reducing the shock period (Fig. 4). Since phagocytic activity is generally impaired far beyond the clinical aspect of shock, it is our opinion that the period of RES depression is the best expression of the true duration of a shock period.

ANESTHETICS

Closely related to surgery is another group of substances, the anesthetics and analgesics. Today, fluothane (Halothan) and nitrous oxide appear to be an ideal combination for long lasting narcosis with little toxicity and few incidents. Since we found postoperatively in patients often a severe RES-depression, which was not related to the stress of operation alone, several anesthetics were examined in mice. The animals underwent a narcosis of 1 hr duration and were subjected thereafter to carbon clearance tests. The marked depression after fluothane narcosis (Fig. 5) could be confirmed in patients; pentrane, ether and nitrous oxide on the other hand, did not effect the RES function.

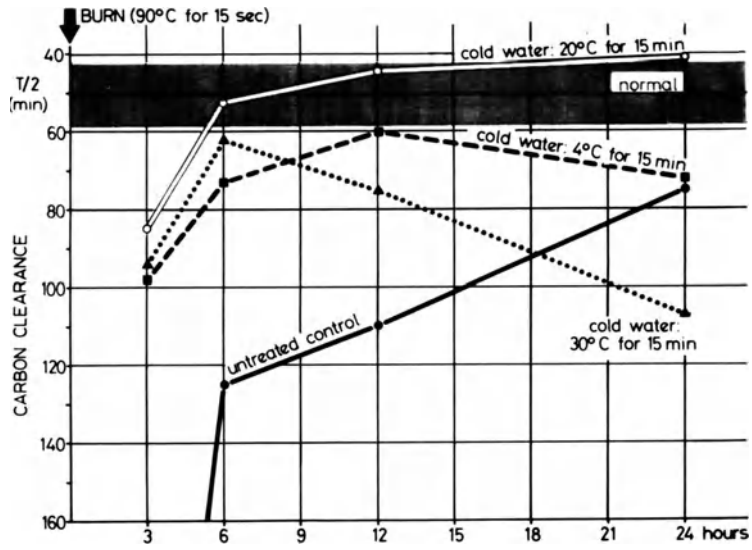

Figure 4: RES-depression after a severe scald lasts in untreated rats and mice for 24 hours; in cold water treated animals only for 6 hours.

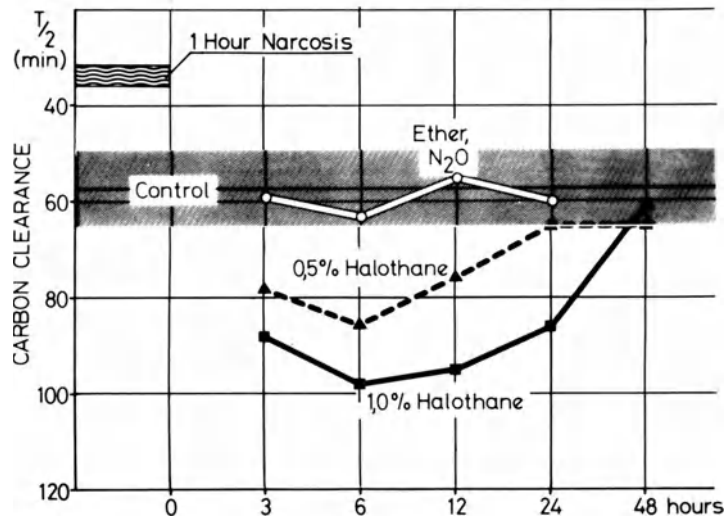

Figure 5: Phagocytic activity is still depressed 24 hours after a one-hour narcosis with fluothane in mice.

A single injection of the maximum daily dose of morphine depressed phagocytosis in mice for 6 hours, while the injection of pentobarbital (Nembutal) caused the strongest depression first after 6 hours (Fig. 6).

This points to a more complicated mechanism of action, presumably related to an impaired energy exchange with the parenchymal liver cell. While Dolantin, for instance, a synthetic morphine derivative, effected a short lasting stimulation of phagocytosis, intravenously injected alcohol stimulated only in low doses but depressed RE functions after a single high dose. The depressive effect of all drugs so far tested was dose dependent and lasted usually for 6 to 24 hours; it could not be increased further by repeated injections, however.

RES-STIMULATION

In animals, a variety of so-called RE stimulants have been examined so far, but their effect often relies on a proliferation of RE cells in liver, spleen, and lung (2,3). Among those substances which are advertised in Germany as stimulants of host defense, only aristolochia acid (Tardolyt) - a plant extract Hippokrates already mentioned for this purpose - proved to have a

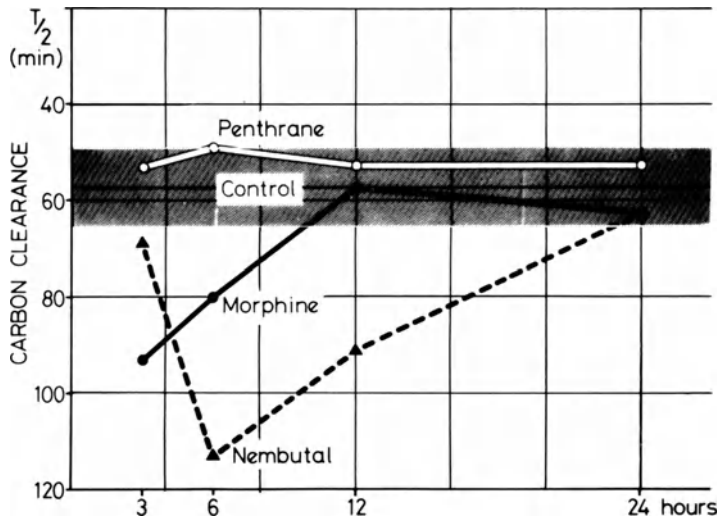

Figure 6: A single injection of Pentobarbital (Nembutal) or morphine depresses phagocytic activity for 12 or 6 hours respectively.

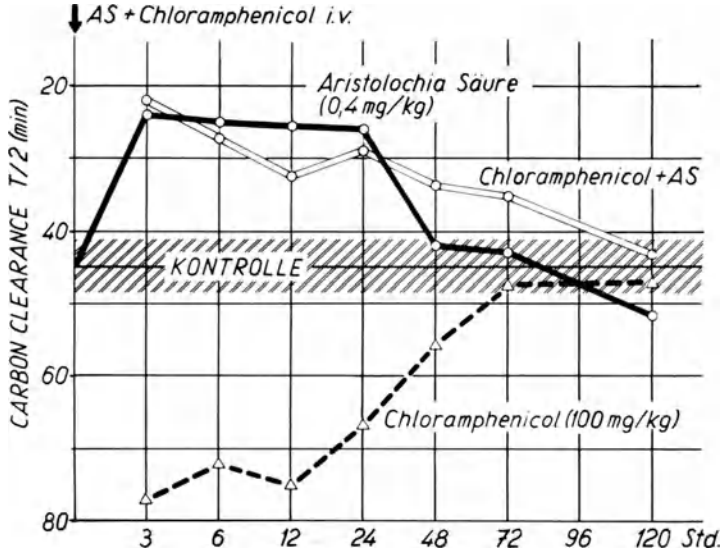

Figure 7: The RE-stimulant aristolochia acid abolishes the RE-depressing effect of chloramphenicol. This might be a useful combination.

stimulating effect on the single RE cell. Phagocytic activity was increased for about 24 hours after one injection in mice and could be held in this state for many days by daily repetitions. Histologic sections revealed more and stronger loaded cells, without signs of cell proliferation.

The RE depressing effect of certain drugs could be abolished by the application of a stimulating agent at the same time (Fig. 7). Since there is little explanation for the mode of action, one has to assume that only a certain percentage of RE cells, f.i. 40% may be involved in phagocytosis under normal circumstances, and that this percentage of RE cells will be increased up to 100% by RE stimulants or antigenic and infectious agents. Even then, a lasting "blockade" of the RES will not be possible, since there are other potential endothelial cells which are able to take over the duties of the normally functioning RE cells. Therefore, RE depression by drugs will be dangerous only in patients who have already used their reserves by severe trauma or long lasting toxemia and are subjected to an unwitting additional assault on their resistance.

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QUANTITATIVE STUDY OF SEX DIFFERENCE IN RE PHAGOCYTOSIS

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The reticuloendothelial system acts a prominent part in non-specific and specific body defense. One important function consists in the clearance of foreign particles by phagocytosis from the blood stream, from the lymph and the connective tissues. In the phagocytic process both the free and fixed cells are involved.

The clearance of particulate matter artificially brought into the blood stream, its accumulation in the different organs and further fate in the body are generally employed as an available model of phagocytic function. In such model experiments, the homeostatic importance of the macrophages is demonstrated by their permanent functional phagocytic state and high metabolic activity. If the foreign substances are metabolizable and antigenic, phagocytosis leads to non-specific defense (1) and production of specific antibodies, the mechanism of which has not yet been fully clarified (2,3).

RES function is controlled by sex-linked factors in some strains of mice. It has been noted by Stern and Davidsohn (4) that C 57 BL female mice possess higher hemolysin titers than do males. Recently Kongshavn and Bliss (5) have shown, that after treatment with anti-mouse thymocyte serum the increase in survival time of grafts on the host is prolonged in the male mice when compared with female mice. Generally,the female mouse seems to be more resistant to various forms of disease than the male mouse (6-8). This difference is correlated with the enhanced immunological responsiveness of female mice. Nicol et al. (9) have postulated that estrogens are the principal natural stimulants of body defense and that sex difference of resistance

may be caused by the action of sex hormones on the macrophages of the RES. In the female mouse, the phagocytic activity varies at different stages of the estrous cycle. Galton (10) has found that the phagocytic activity of a group of randomly selected female mice was significantly higher than of male mice. It has been suggested that 17 β-estradiol is the main compound among the natural estrogens controlling RE function. Treatment of male mice with 17 β-estradiol causes a marked increase of the phagocytic activity of the RES (11). In this case, the functional basis of the pharmacologically induced phagocytic increase mainly is an enhancement of the cell number in the liver and spleen (11,12). However, no histological observations are available that demonstrate the action of the endogenous (native) estrogens on the RES of mature female mice.

Therefore, the present work is concerned with a quantitative examination of the phagocytizing RE cells in the liver of normal mature male and female mice. Above all, the extent of the sex difference of the phagocytic activity had to be investigated. Furthermore, it was studied whether the sex difference was caused by a difference in cell activity or by a difference in the number of the macrophages in male and female mice. The majority of all active macrophages participate in phagocytosis only when large amounts of test colloid are brought into the blood stream (13). Therefore, we used 5 different carbon doses. The accumulation of the carbon particles in the liver and spleen was examined by light microscopy. As secondary sex-linked characters manifest only after sexual maturity and, therefore, are closely connected with growth, 4 week old mice of both sexes were investigated for comparison.

The phagocytic activity of the RES can be influenced either by an alteration of the activity of the single cells or by a change in the cell number. In order to study these two factors, we produced proliferation of RE elements by 17 β-estradiol (12) or stimulation of their activity by a lipopolysaccharide (14). It was our assumption that this difference in the mode of action on a histological basis together with the kinetics of the clearance process may give further information concerning the nature of the mechanism that causes the sex difference of phagocytic function.

MATERIAL AND METHODS

Intact male and female mice (NMRI) 4 and 8 weeks old, were used for these investigations. Groups of 5-6 animals were randomly selected.

Phagocytic activity. The phagocytic activity was evaluated by means of the carbon clearance test (Carbon suspension No. C11/1431 a, G.Wagner, Hannover, Germany). With reference to the procedure evolved by Biozzi et al. (15), the lowest dose of carbon was adjusted to body weight. In this experiment the standard dose of 16 mg carbon per 100 g body weight (about 4.2 to 4.8 mg carbon per animal) was injected intravenously (tail vein). The other experiments were performed with 4 higher carbon doses. In this series, doses of 8, 12, 16 and 20 mg of carbon each suspended in 0.25 ml gelatin (2-5%) were injected per mouse without regard to the body weight of the animals, because no correlation between body weight and the rate of carbon clearance was found by Fred et al. (13,16). Within 66 minutes after the carbon injection, several blood samples of 25 µl were drawn from the retro-orbital plexus. The carbon concentration of the blood was measured by photometry in the red light (691 nm).

Kinetics of carbon phagocytosis. The clearance process of different carbon doses is not uniform (13,17-20). At low doses of carbon the clearance curves are nearly exponential, but when large amounts of carbon are used, there is an initial linear clearance rate that changes into an exponential one when the carbon concentration in the blood is decreased. The length of the single segments depends on the dose injected and the phagocytic state (ability) of the RES. Using the lowest dose of 16 mg carbon per 100 g body weight, statistical analysis was based on the calculation of the phagocytic indices K and α (15). With regard to the heterogenous kinetics of the clearance of large amounts of carbon in the experiments with 8, 12, 16 and 20 carbon mg per animal, the absolute amount of carbon taken up by the RE cells within 66 minutes was calculated (a) for 1 ml of blood and (b) for the whole blood volume. The carbon concentration per 1 ml blood was estimated by means of a calibration curve for serial dilutions of a stock suspension of 12 mg carbon per 1 ml solution. The total blood volume containing the carbon can be estimated by extrapolating the disappearance curves to the Y axis. As the concentration at zero time is known from the calibration curve and the amount of carbon injected, the blood volume containing the carbon can be calculated. The "carbon distribution space" calculated in this way (21) is in a good agreement with the blood volume evaluated by help of several dyes normally used (22).

Pharmacological stimulation of the RES. In 8 week old male mice the RES was stimulated by 17 β-estradiol and the lipopolysaccharide Pyrexal. 17 β-estradiol was dissolved in sesame oil. One mg per 20 g body weight in 0.1 ml oil was injected i.p. once daily for 3 days. The phagocytic activity was measured on the 4th day after treatment had started. The lipopolysaccharide

Pyrexal was administered intravenously at a dose of 10 μg per 20 g body weight in 0.2 ml isotonic saline. In this experiment the phagocytic activity was measured 2 days after the injection.

Histological observations. The liver of some male and female mice was fixed in Bouin-Allen's fluid, and sections were cut 7μ thick and stained with hemytoxylin and eosin. The carbon-containing macrophages were counted in representative circular units 0.25 mm in diameter (a) from the central regions of the liver lobules and (b) from the perilobular zone.

Statistics. The significance of differences between means was calculated by Student's t-test and was based on the 95% confidence limit. Variances were compared using the F-test.

RESULTS

Comparison of Phagocytic Activity in Infantile Male and Female Mice

The 4 week old mice were injected with 8, 12, 16 and 20 mg carbon per animal (about 35 to 87 mg carbon per 100 g body weight). The initial carbon concentration in the blood at zero time increased in proportion to the dose injected (Table I). As there was no difference between sexes, these data show that the blood volume of the male and female mice did not differ. Corresponding to the increment in the initial carbon concentration caused by the 4 different doses, the amount of carbon withdrawn from 1 ml blood and from the whole blood was increased. The data show that there were no differences in carbon uptake between sexes except for the 20 mg dose. The amount of carbon withdrawn from the whole blood was 10% higher in the females than in the males ($p<0.05$). No sex difference was found in the weight of liver and spleen).

Sex Difference in Phagocytic Activity of Mature Male and Female Mice

Low dose of carbon: 16 mg per 100 g body weight. In 8 week old mature female mice, the standard dose of 16 mg carbon per 100 g body weight was cleared from the blood by a significantly higher rate than in the male animals (Table II). The difference of 47% ($p<0.001$) between the conventional K values is in agreement with the findings of Galton (10). The total amount of carbon taken up within 20 minutes was also

Table I. Dose dependence of carbon uptake in the RES determined by 8, 12, 16 and 2o mg carbon per animal and organ weight of 4 weeks old male and female mice[a]

Carbon dose mg	Sex[b]	Initial carbon concentration mg/ml blood[c]	Carbon uptake from blood as mg per 1 ml	Carbon uptake from blood as mg per Whole blood	Weight Body g	Weight Liver g	Weight Spleen mg
8	Male	4,64±o,26	3,21±o,33	5,53±o,39	22,7±1,3	1,2o±o,12	1o8±16
	Female	4,92±o,43	3,25±o,38	5,3o±o,48	22,o±2,o	1,13±o,o7	1o6±15
12	Male	7,o3±o,69	5,11±o,33	8,79±o,97	22,3±1,6	1,32±o,18	112±2o
	Female	7,25±o,19	5,15±o,35	8,52±o,49	21,9±1,2	1,31±o,o4	1o9±15
16	Male	9,16±o,61	5,79±o,93	1o,1o±1,33	23,4±o,8	1,28±o,o9	123± 9
	Female	8,74±o,68	5,63±o,53	1o,32±o,53	22,9±1,3	1,27±o,12	12o±18
2o	Male	1o,45±o,85	7,o7±o,51	13,56±o,72	23,8±1,6	1,44±o,1o	157± 6
	Female	1o,17±o,62	7,57±o,75	14,86±o,76	23,1±2,2	1,32±o,19	13o±28

[a] Mean ± SD.

[b] 5-6 animals per group.

[c] Calculated by extrapolation of the clearance curves on the Y axis.

[+] $p < o,o5$.

Table II. Sex difference of RE phagocytic activity of 8 week old mice confirmed by a low dose of 16 mg carbon/100 g body weight.[a]

Sex[b]	Phagocytic Activity: Phagocytic Index K x 10^3	Phagocytic Activity: Phagocytic Index α	Phagocytic Activity: Carbon uptake mg/20 min	Weight: Body g	Weight: Liver Spleen g
Male	10.9 ±1.8	3.62 ± 0.36	1.87 ± 0.19	29.3 ±1.3	1.81 ± 0.13
Female	16.0 ±2.2	4.14 ± 0.32	2.21 ± 0.25	26.0 ±1.1	1.59 ± 0.12

[a] Mean ± SD.

[b] 12 animals per group.

$p<0.001$.

significantly higher in the females than in the males. There were no significant differences between the variances of both sexes. The rise in the corrected phagocytic index α of the female mice demonstrates that the phagocytic activity per unit tissue was higher than in the male mice. This was supported by the findings that the combined weight of liver and spleen in the females was lower than in males.

Large doses of carbon: about 26 to 66 mg per 100 g body weight. The initial carbon concentration in the blood increased corresponding to the dose injected (Table III). The increase was the same in male and female mice demonstrating that the blood volume was not different. The amount of carbon cleared within 66 minutes increased in both sexes, corresponding to the initial carbon content in the blood. However, the phagocytic activity was significantly higher ($p<0.05$ - $p<0.001$) in the females than in the males without regard to whether the carbon uptake was based on 1 ml blood or on the whole blood. No difference in liver weight was found between male and female mice. However, the spleen of the female mice was somewhat enlarged.

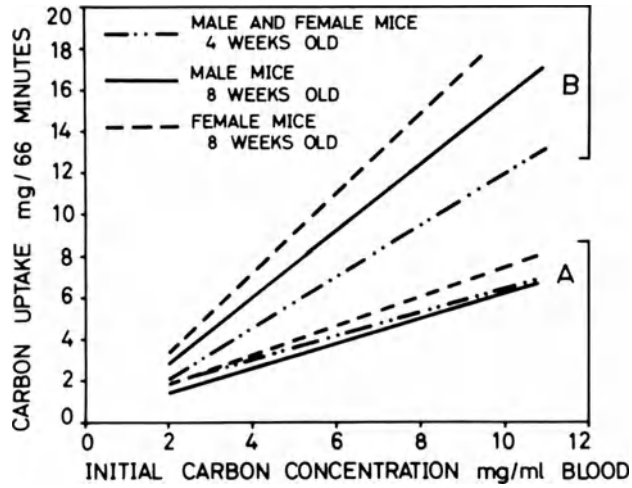

Fig. 1. Dependence of carbon uptake on the initial carbon concentration in the blood of 4 and 8 week old mice. Amount of carbon withdrawn from 1 ml blood (A) and the whole blood (B).

The Influence of Growth on RE Function

Sex characters that are secondary manifest for the first time with maturation. The same must be true for a phagocytic mechanism that is controlled by hormones of the gonads. As maturation is closely connected with growth, this parameter per se might influence the phagocytic function of the RES. Fig. 1 demonstrates the changes in the phagocytic activity of male and female mice during the period of growth from week 4 to week 8 after birth. As was demonstrated in Table I, there were no sex differences in the phagocytic activity of the 4 week old immature mice except after 20 mg carbon. Therefore, the mean values of the 4 week old male and the female mice were combined for presentation. The regression lines in Fig. 1A show that the phagocytic activity of the 4 week old mice (male and female) was somewhat higher than that of the 8 week old male mice if the carbon uptake was based on 1 ml blood. This is in agreement with the findings that the K value of rats decreases slowly with age (23,24). On the other hand, the amount of carbon withdrawn from the whole blood was significantly higher in the 8 week old males than in the 4 week old males and females (Fig. 1B). This increase in the activity of about 32% (within the whole range of the initial carbon concentration) ran parallel with a 33% increase

Table III. Dose dependence of carbon uptake in the RES determined by 8, 12, 16 and 2o mg carbon per animal and organ weight of 8 weeks old male and female mice[a]

Carbon Dose mg	Sex[b]	Initial carbon concentration mg/ml blood[c]	Carbon uptake from blood as mg per 1 ml	Carbon uptake from blood as mg per Whole blood	Weight Body g	Weight Liver g	Weight Spleen mg
8	Male	3,72±o,17	2,26±o,14 ‡	4,85±o,18 ‡	31,6±1,o	1,72±o,11	1o7±9
	Female	3,94±o,27	3,11±o,13	6,35±o,39	29.8±1,3	1,66±o,16	134±29
12	Male	4,93±o,36	3,23±o,35 ‡	7,86±o,57 ‡	33,o±1,7	1,78±o,1o	135±14
	Female	5,38±o,35	4,42±o,23	9,86±o,59	3o,o±1,o	1,66±o,1o	18o±27
16	Male	6,6o±o,3o	4,29±o,56 +	1o,42±1,61 +	32,5±1,o	1,91±o,13	136±18
	Female	6,37±o,41	5,o4±o,37	12,68±o,69	31,4±1,4	1,75±o,16	168±32
2o	Male	7,78±o,46	4,67±o,5o ‡	11,99±o,95 ‡	33,o±1,7	1,8o±o,12	125±21
	Female	8,15±o,31	6,o7±o,58	14,88±1,18	31,1±1,4	1,87±o,19	165±13

[a] Mean ± SD.

[b] 5-6 animals per group.

[c] Calculated by extrapolation of the clearance curves on the Y axis.

+ $p<o,o5$, ‡ $p<o,o1$, ‡‡ $p<o,oo1$.

in the blood volume from about 1.8 ml for the 4 week old mice to 2.4 ml for the 8 week old male mice and an enlargement of the liver of about 38% from 1.3 to 1.8 g. The RE function of the 8 week old female mice was influenced by the same parameters of growth. However, there was another factor stimulating in addition the phagocytic function in comparison with the 8 week old male mice (Fig. 1A and B).

Influence of Cell Activation and Macrophage Proliferation on the Kinetics of Carbon Clearance

The higher phagocytic state of the RES of the adult female mice caused a fast initial decline of the clearance curves within 20 to 30 minutes after each of the 4 different large doses. The following segments of the curves showed an essentially lower activity (Fig. 2A). In opposition to this heterogenity, the clearance curves of the male mice were nearly uniform (Fig. 2B).

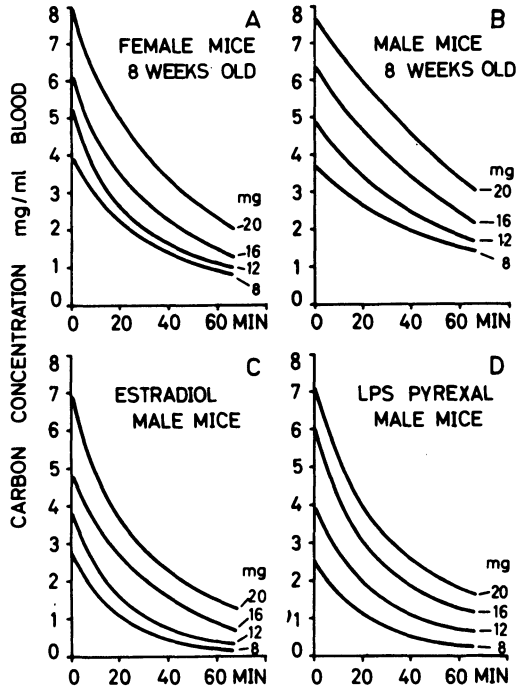

Fig. 2. Kinetics of blood clearance after different carbon doses in adult normal female (A) and male mice (B) and in male mice treated with estradiol (C) or the lipopolysaccharide Pyrexal (D). Estradiol (3 x 1 mg) mainly caused proliferation, whereas Pyrexal (10 g) produced cell stimulation (see Table IV). Carbon doses: 8, 12, 16 and 20 mg per animal.

Fred et al. (20) have recently demonstrated that colloid clearance changes from a linear rate into a quasi-exponential one and at last into an exponential phase as the number of particles in the blood becomes (is) too small relative to the binding sites on the RE cells. This led us to the assumption that there could have been a greater number of macrophages in the adult female mice than in the male ones.

Treatment of mice with estradiol causes a marked increase of the number of phagocytizing RE cells (11,25). Thus, estrogen-treated animals may be useful to investigate the influence of an increase in the macrophage population or in the binding sites for particles on the shape of the clearance curves (13,20). After the injection of 3 x 1 mg 17 β-estradiol, the number of the carbon-containing macrophages was increased in the perilobular zone of the liver lobules. Moreover, an enlargement of the liver and spleen was observed (Table IV A). From these data the total number of phagocytizing cells in the liver of the estrogen-treated animals was calculated to be 150% of the oil-control group. The shape of the clearance curves of the estrogen-treated males (Fig. 2C) was nearly the same as that of the non-treated 8 week old females. The same kinetics of carbon clearance was caused, however, in males by the lipopolysaccharide Pyrexal (Fig. 2D) which, in contrast to estradiol, caused only stimulation of the preexisting cells without an increase in the number of phagocytizing cells and (or) enlargement of the liver (Table IVB). The results demonstrate that large doses of carbon were no longer saturating not only in the case of increased numbers of phagocytizing cells but also if the activity of the phagocytes already existing in the liver was stimulated pharmacologically. According to these results, it was impossible to decide only by help of the clearance curves of the normal females whether there was an increase in the cell number in comparison to the normal males or a higher activity of the cells. In order to answer this question, the carbon-containing cells were counted in liver sections of both female and male mice. The number of the active macrophages per liver lobule was slightly increased (+11%) in the female mice (Table V). However, the liver weight of some female animals selected for histological studies was lower than that of the male ones and therefore, the absolute cell number must have been nearly the same in the female mice as in the male mice or even smaller. As the carbon uptake was higher in the females than in the males, these findings led to the conclusion that the sex difference in phagocytic activity is caused mainly by an increased phagocytic activity of the "responsive" macrophages in female mice and not by a rise in their number.

Table IV. Carbon-containing macrophages in liver sections and weight of liver and spleen after treatment with 17β-estradiol (A) and LPS Pyrexal (B)[a]

	Treatment	Macrophages[b] Centrilobular	Macrophages[b] Perilobular	Weight[d] Liver g	Weight[d] Spleen mg
A.	Sesam Oil (Controls)	53 ±7	69 ±8	1,80 ±0,19	138 ±24
	Estradiol 3 x 1 mg	54 ±9	102 ±8	2,16 ±0,24	181 ±40
B.	Saline (Controls)	47 ±8	63 ±7	2,04 ±0,23	139 ±25
	Pyrexal 10 µg	45 ±6	68 ±9	1,98 ±0,20	240 ±35

[a] Mean ± SD.

[b] 7-µ liver sections, 20 - 30 fields of 0,25 mm in diameter per group.

[c] 17-21 animals per group.

Table V. Carbon-containing macrophages in liver sections and liver weight and phagocytic activity of 8 weeks old male and female mice[a]

Sex[b]	Macrophages[c] Centrilobular	Macrophages[c] Perilobular	Liver weight g	Carbon uptake mg per 1 ml	Carbon uptake mg per whole blood
Male	52 ±9	61 ±8	1,77 ±0,13	2,68 ±0,51	6,39 ±1,67
Female	60 ±10	65 ±6	1,65 ±0,14	3,74 ±0,80	7,75 ±1,83

[a] Mean ± SD.

[b] 4 animals per group

[c] 7-µ liver sections, 40 fields of 0,25 mm in diameter per group.

DISCUSSION

Our experimental results confirm the findings of Galton (10) that the phagocytic activity of the RES is higher in adult female mice than in male mice. This sex difference could be demonstrated using several carbon doses between 4 and 20 mg carbon per animal (16 to about 66 mg per 100 g body weight). Absolutely, the difference increased with the dose injected. Calculated on a per cent basis, the phagocytic activity of the female mice remained constant at about 20% above the level of the male mice.

No sex difference was found in 4 week old mice except for the largest dose of 20 mg per animal. The difference was very small and, therefore, it is likely that it was an artifact caused by a carbon dose that was too large for animals of 23 g weight. Using more than 24 mg carbon in mice of 35 g weight, Fred (26) has shown that the carbon particles were not only phagocytized by the RE cells but were also sequestered in capillary and venule emboli, thus leading to an artificial increase in the phagocytic rate. With regard to the smaller blood volume of a 23-g mouse an overload of the blood constituents may be expected at a lower dose level, i.e. at about 20 mg carbon per animal. The time at which the higher phagocytic rate of female mice manifests for all carbon doses tested in these experiments coincides with maturation at the age of about 6 weeks. This is in agreement with the findings of Nicol *et al*. (9), who demonstrated hormonal control of phagocytic activity of female mice. These authors have shown that RE phagocytosis varies according to the different stages during the estrous cycle and that ovariectomy causes a loss of phagocytic peaks during proestrus and metestrus (9,27).

It must be stated, however, that newly published data and some of our own results give rise to the assumption that sex differences in RE and related functions are not exclusively caused by hormonal control. In consideration of the great variations of phagocytic activity during the estrous cycle found by Nicol *et al*. (9), a greater variance in the activity of the females was also expected in our experiments. But this proved not true, and Galton (10) has made the same observations. Furthermore, the mean values of the mature female mice in our experiments were remarkably high. Nicol *et al*. (9) have shown that the phagocytic activity is very low in diestrus during 60% of the estrous cycle. There were two peaks of increased phagocytosis with a duration of only about 25% of the whole length of the estrous cycle.

With regard to our own results, data are very interesting showing that differences in the RE phagocytic activity of rodents may have a genetic background (28) and can also be influenced by

environmental factors (29). Furthermore, remarkable variations in the responsiveness of RE phagocytic function to experimental stimulation were observed in different strains (29). From this point of view, the following must be taken in consideration: a) the basal phagocytic activity that is genetically determined may be different a priori in mature male and female mice of some strains, b) the functional connection between the RE cells and the estrogen level in the blood may be varied in female mice of different strains. This might cause strain-specific different features of the variations of phagocytosis during the estrous cycle. That the sex difference in phagocytosis manifests only at the time of maturation, must not be inconsistent with the assumption of an otherwise genetic control. The ontogeny of estrogen specific receptors in the uterus of the rat for example is subjected to a direct genetic control: it is not dependent, however, on the onset of hormonal release (30). As the liver is no real target organ for estrogenic action with specific receptors (31,32,33), a greater variability in the dependence of RE function on estrogens of different strains of mice is quite possible. This is supported by experimental results of Di Luzio (34), who in contrast to Nicol et al. (9,27), found no influence of ovariectomy on RE phagocytosis. The secondary hemagglutinin response of females of the Balb x C 57 BL strain that is higher than in males, but is not influenced by ovariectomy (6), will be another example for a non-gonadial control of sex difference. In this connection, the fact must be mentioned that estrogenicity and RE stimulating properties of an estrogen act independent and thus can be dissociated (27).

Besides the estrogenic aspect, the possibility must be taken into account that testosterone and its natural derivatives may cause the sex difference in phagocytic activity. That means that the phagocytic activity of the mature females must be considered as the basal activity of adult mice, that is not influenced by hormones. However, there is no evidence in the literature for a significant action of endogenous testosterone on the phagocytic activity of the RES. Furthermore, Nicol et al. (11,35) have found no influence of injected testosterone even when the dosage was very high in comparison with physiological levels. In connection with our experiments, the fact is more important that orchiectomy did not influence the phagocytic activity of mature male mice (23,34). The lack of a decisive hormonal control of RE function by the gonads of the mature males was confirmed by our own results. When the comparison of the phagocytic activity of the 4 week old male mice with that of the 8 week old male mice was based on the dose dependence of carbon uptake, we could find only a significant positive influence of growth on the phagocytic activity. However, there was no evidence for an additional hormonal interference.

In summary, the results of these investigations demonstrate that the sex difference in the phagocytic activity was caused by the fact that the phagocytic mechanism of the female mice is not only influenced by the same parameters of growth that likewise existed in male mice, but that there was an additional functional increase at the time of maturation. The higher phagocytic state of the females must have been caused by stimulation of the single cells rather than by enhancement of their number as was shown by the cell counts in the liver. Splenomegaly in the females was small and could play no significant role among the causes of the difference. There is evidence nowadays that differences in the RE function of male and female mice are not only controlled by hormones of the gonads but may also be influenced at least by other factors on the basis of a genetic background.

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THE TEMPORARY SEQUESTRATION OF C3-COATED RED CELLS (EC43) IN THE RES OF THE RABBIT: A MECHANISM FOR THE NON-LYTIC DAMAGE OF RED CELLS BY COMPLEMENT

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The removal of inert particulate material or opsonized bacteria and red cells from the circulation at sites in the RES is normally a permanent and irreversible event (1, 2, 3, 4). However, red cells which have fixed complement without proceeding to lysis may be an exception to this rule and complement-coated red cells can under certain conditions undergo only temporary sequestration in the RES. The present report describes a possible mechanism for this temporary sequestration; a mechanism which causes damage to the red cell without lysis and is related to the activity of membrane-bound C3, the third component of complement.

Table 1. The Reactions of Rabbit Red Cells with Anti-I Cold Agglutinins and Rabbit Sera

A. $E + A_{(IgM\ cold\ agglutinin)} \xrightarrow{+\ normal\ rabbit\ serum} EA_{(IgM)}C1\text{-}9 \longrightarrow E^*$ (lysis)

B. $E + A_{(IgM\ cold\ agglutinin)} \xrightarrow{+\ C6\text{-}deficient\ serum} EA_{(IgM)}C1\overline{42}3$

C. $E + A_{(IgM\ cold\ agglutinin)} \xrightarrow{+\ C3\text{-}9\ depleted\ serum} EA_{(IgM)}C1\overline{42}$

Rabbit red cells, which possess the big-I antigen (5), fix complement and lyse (Table 1,A) in the presence of normal rabbit serum and human cold agglutinin of anti-I specificity (6). If, however, serum from rabbits homozygous for C6-deficiency is substituted for normal rabbit serum in this system (Table 1,B) the red cells fail to lyse and a red-cell complement intermediate in the form $EA_{(IgM)}C1\overline{42}3$ is generated (7), the later complement components including C5 not being fixed in the absence of C6. By a similar argument, rabbit red cells incubated with serum depleted of C3-C9 by cobra-venom factor (8) will fix the early components of complement in the presence of human cold agglutinin, but in this case fail to fix C3 (Table 1,C).

Table 2. The Decay of Complement Intermediates during the Removal of the Cold Antibody

C6-deficient system

$$EA_{(IgM)}C1\overline{42}3 \xrightarrow[\text{Wash x 6}]{40^{\circ}C} EC43$$

C3-depleted system

$$EA_{(IgM)}C1\overline{42} \xrightarrow[\text{Wash x 6}]{40^{\circ}C} EC4$$

Following washing to remove the cold agglutinin the respective complement intermediates become, in the case of the C6-deficient system, EC43,(C1 is removed with the antibody and the $C\overline{42}$, convertase, site decays to C4), and in the C3-C9 depleted system, EC4. These steps are shown in Table 2. Red cells prepared in the form EC43 by the technique outlined above,showed marked fixed-C3 activity. They agglutinated intensely with conglutinin, immunoconglutinin and with anti-C3 serum and showed positive immune-adherence with rabbit platelets *in vitro*; in addition EC43 cells adhered to rabbit macrophages and neutrophils, underwent distortion and contraction (Fig 1) and in some cases were ingested.

In marked contrast, EC4 cells did not show fixed-C3 activity; they failed to agglutinate with conglutinin and immunoconglutinin, did not react by immune-adherence nor did they attach to rabbit neutrophils or macrophages.

Neither of these two complement-red cell intermediates, however, lysed in fresh normal serum due to decay of the $C\overline{42}$, convertase site. The *in-vivo* behavior of EC43 cells is outlined in the following section*.

* A detailed description of this section has been published by Brown, Lachmann & Dacie, 1970. (Ref. 11)

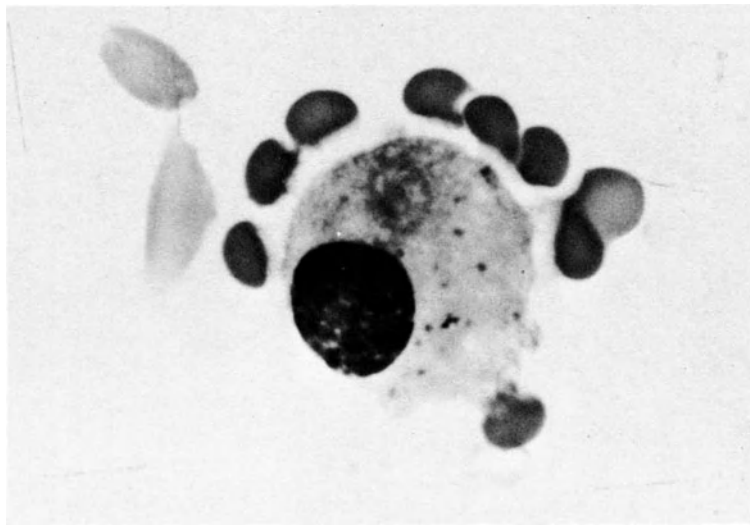

Fig 1. Distortion and contraction of red cells in the form EC43 following adherence to rabbit lung macrophages in-vitro.

Fig 2 illustrates the patterns of survival of autologous chromium-labelled EC43 cells following intravenous injection into rabbits. Immune adherence between the injected red cells and circulating platelets, neutrophils and monocytes occurred immediately, and the leucocyte-red cell or platelet-red cell complexes rapidly disappeared from the peripheral blood. This occurred in a single exponential manner with a $t_{\frac{1}{2}}$ of $1\frac{1}{2}$-4 minutes. Minimal values were reached within 8-15 minutes post-injection and then the red cells returned slowly to the circulation again in an exponential manner with a "build-up" of $t_{\frac{1}{2}}$ of 25-100 min. When small amounts of EC43 cells were injected ($\leq 10^9$/kg) there was a significant permanent loss of cells, with 50% or less of the predicted number returning to the circulation (curve X).

In marked contrast, EC4 cells following intravenous injection did not adhere to platelets or leucocytes in vivo; were not temporarily sequestered and survived normally during the period of observation. (Fig 3).

Following the intravenous injection of chromium-labelled EC43 cells into rabbits, radioactivity over the liver, as detected by surface counting, rose, then subsequently fell in a reciprocal manner to the changes in peripheral blood red cell radioactivity indicating temporary sequestration in this organ. The high level of maximal sequestration in the liver (almost 90% of the injected dose of labelled EC43 cells) and subsequent decline with time were confirmed by killing rabbits at intervals and counting

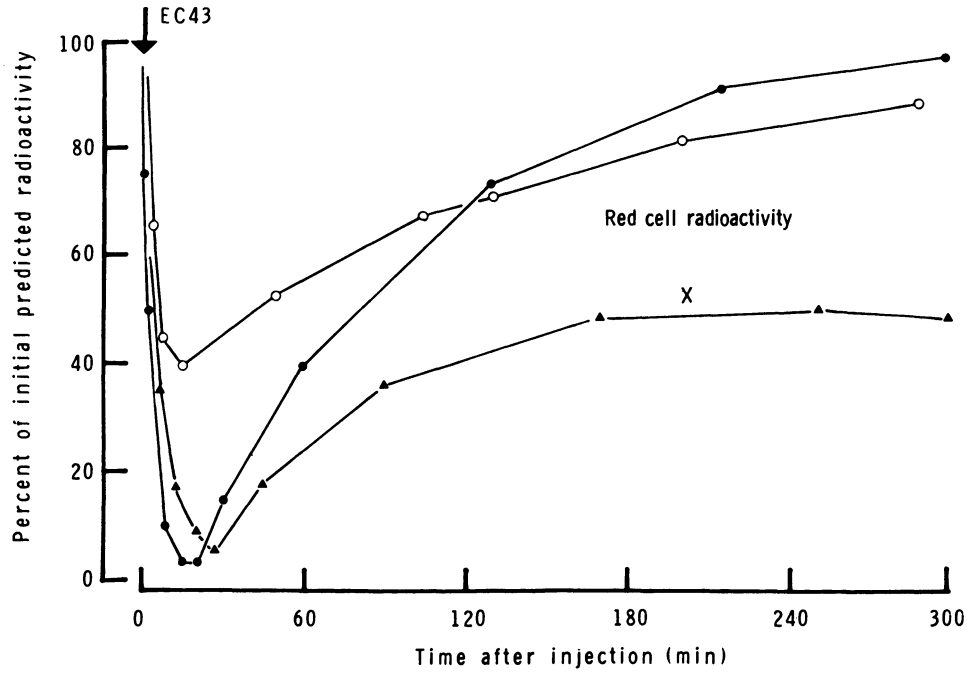

Fig 2. In vivo survival of autologous chromium-labelled EC43 cells following intravenous injection into rabbits.

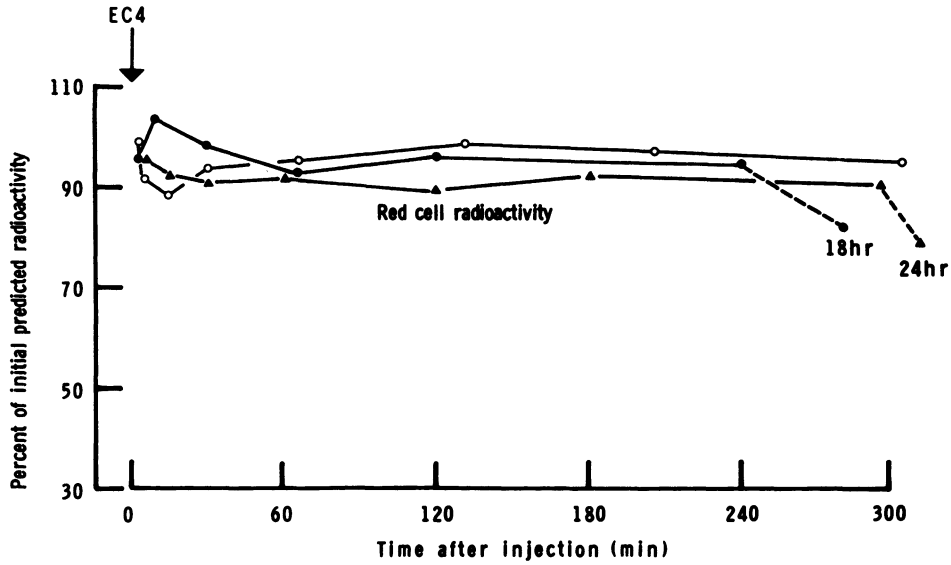

Fig 3. In vivo survival of autologous chromium-labelled EC4 cells following intravenous injection into rabbits.

the radioactivity in different organs. The lungs contained about 10% of the sequestered red cells and the spleen 2-4%. Examination of the liver either by histological section or by imprint preparations 8-15 minutes after injecting EC43 cells intravenously showed red cells adhering to Kupffer cells with occasional ingested red cells visible. (Figs 4a and 4b).

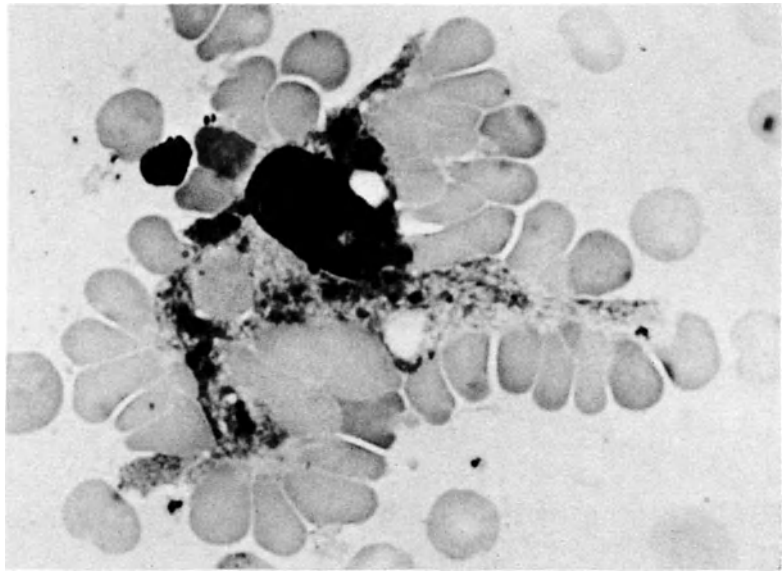

Fig 4 (a). Kupffer cell showing EC43 cells attached and in some instances ingested.

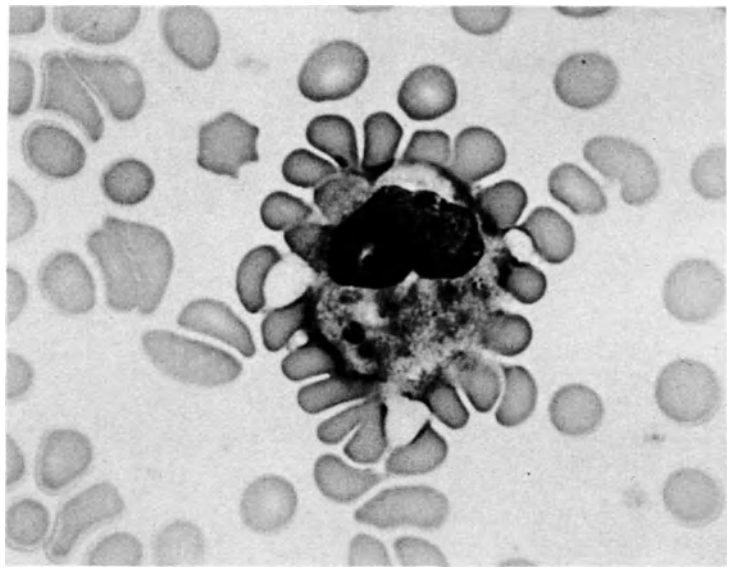

Fig 4 (b). Kupffer cell showing attached and distorted red cells.

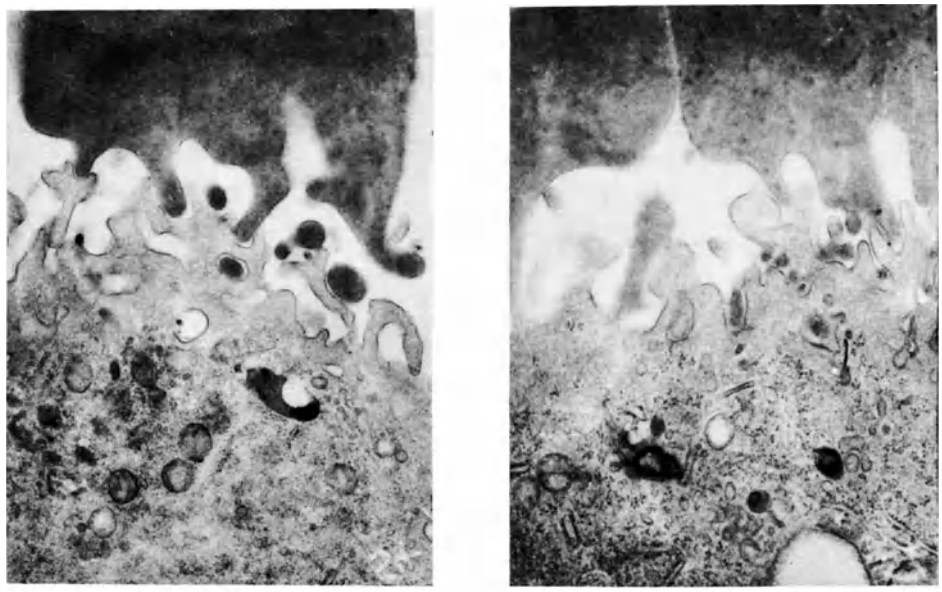

Fig 5 (a and b). Distortion and indentation of the red cell membrane (at top) at the point of attachment to the macrophage (EM section; original magnification X100,000)

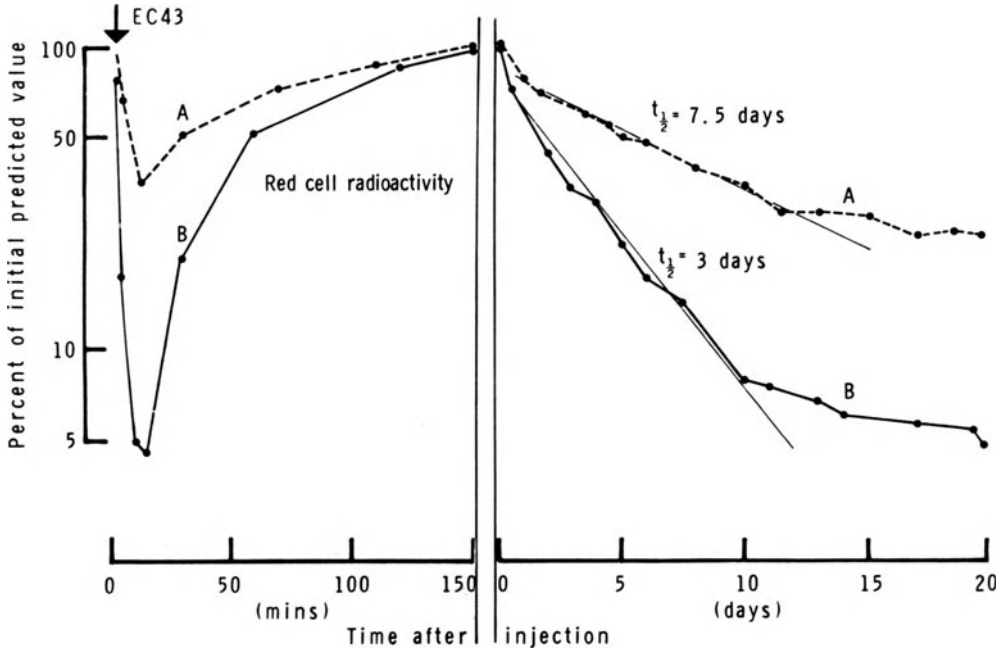

Fig 6. Survival of chromium-labelled EC43 cells in two rabbits A and B, during the acute sequestration phase (left) and during the subsequent phase of slow permanent removal (right). Note log scale on the ordinate.

The nature of the contraction and distortion of the complement-coated red cells while they were in contact with Kupffer cells may be related to a similar appearance found when EC43 cells were incubated in vitro with rabbit lung macrophages. Examination of EM-preparations from such material showed marked indentation and villus distortion of the red cells at the point of attachment to the macrophages (Figs 5a and 5b). Inspection of serial sections gave the impression that endocytosis of small vesicles of membrane was taking place at the point of contact, so that the red cells were, in effect, losing surface area to a greater degree than they were losing volume. This might account for the denser spherical appearance of such macrophage-bound red cells when viewed by light microscopy as described by other authors (9, 10).

We have previously shown (11) that Kupffer cells examined 2-4 hr after the rabbit received EC43 cells were devoid of attached or phagocytosed red cells and that spherocytes, presumed to be the previously sequestered red cells, were visible in the peripheral blood at this time. This return of sequestered EC43 cells to the circulation was related in time to the decline of the antigenic and functional activity of red-cell bound C3 in vivo.

The ultimate fate of chromium-labelled EC43 cells after temporary sequestration is illustrated in Fig 6. The red cells were slowly but permanently removed in an overall non-linear manner, though with a log-linear component of $t_{\frac{1}{2}}$ 3-10 days. Counting of organs for ^{51}Cr at the end of this period of slow disappearance showed that counts/g in the spleen were 90-200 times those in the liver, suggesting highly selective irreversible removal by the spleen. This was in contrast to the initial reversible sequestration which occurred predominantly in the liver.

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FUNCTION AND IMPORTANCE OF THE RES DURING PERFUSION OF THE LIVER

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Conservation of the liver and maintaining the integrity of the RES cells becomes of particular importance in temporary extracorporal homologous or heterologous liver substitution in the treatment of hepatic coma and in bridging rejection crises after transplantation of organs. Since the success of the given therapeutic possibilities depends largely on the function of the RES cells and particularly on their detoxicating properties, we have set ourselves the task of investigating the phagocytic activity of the RES cells in the liver during conservation and prolonged perfusion.

First of all one should examine the question why, in perfusion with homologous or heterologous blood, repeatedly reported outflow block or pressure increase in the supply system occurs, leading the development of edema of the liver, dependent on time. Since such an outflow block is only very slight or not observed at all with perfusion media which do not contain hemoglobin, it is necessary to establish whether there is a connection between the phagocytic activity and the falling off of the flow, which can be related to changes in the RES cells.

METHOD

After laparotomy, the liver was isolated *in situ* after cannulation of the hepatic artery and portal vein and cooled with a wash-out solution 0 - 5°C for 15 - 20 minutes until a temperature of 18 - 20°C was attained, to prevent primary damage from hypoxia. The flow rate was at least 0.5 ml/g/min for the heaptic artery and 1.5 ml/g/min for the portal vein. It was only allowed to change to

FIG. 1. Stored liver in the perfusion chamber (pig liver).

an erythrocyte-free perfusion media after this temperature had been reached. The liver was stored according to Miller in a plexiglass chamber which permitted the use of an excess pressure of 6 - 8 atmospheres (Fig. 1). During the low flow perfusion, pressure of 0.25 - 1.0 atmospheres was maintained, with rythmic oscillations of 0.5 atmospheres at invervals of 20 minutes. The temperature of the organ fell to 10 - 14°C. The perfusion system is shown in Fig. 2, with the cooling unit, heat exchanger, bubble oxygenator, pulsating arterial pump for the hepatic artery and rotary pump for the portal vein, as well as the venous return pump and perfusion chamber.

The diagram (Fig. 3) shows that glass spirals were introduced into the circulation situated before and after the liver, and Picker dual flowmeters fixed into the measuring heads of the scintillation counter. Colloidal radioactive gold was used as the material for the phagocytosis as it fulfills most easily the conditions laid down by Benacerraf for phagocytosed substances.

The fall in plasma concentration of the test substance as a function of time follows the formula

$$C_2 = C_1 e^{-kt} \qquad (a)$$

(Biozzi, Benacerraf, and Halpern).

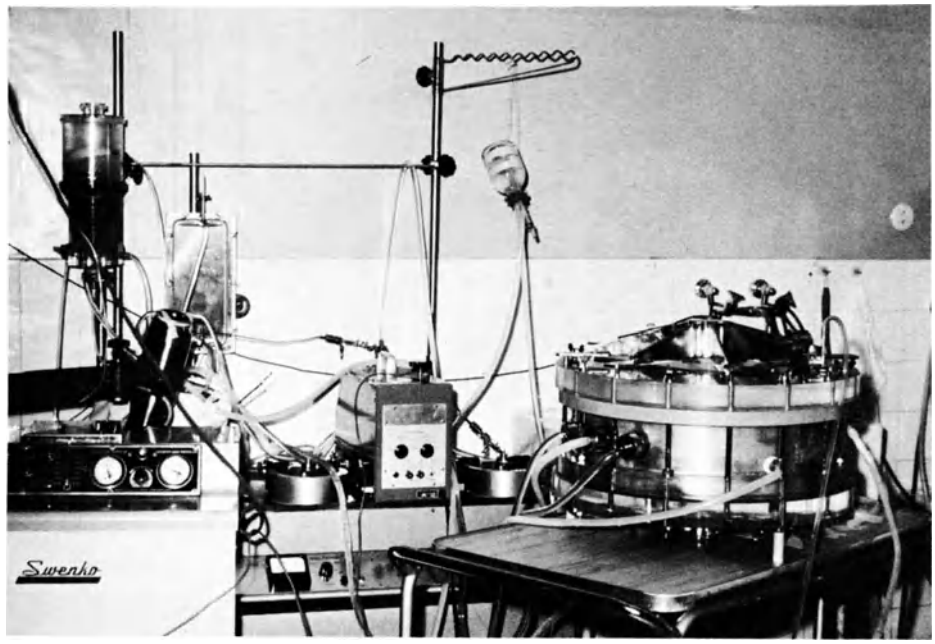

FIG. 2. Perfusion system: Cooling unit, heat exchanger, bubble oxygenator, pulsating arterial and venous (rotary) pump.

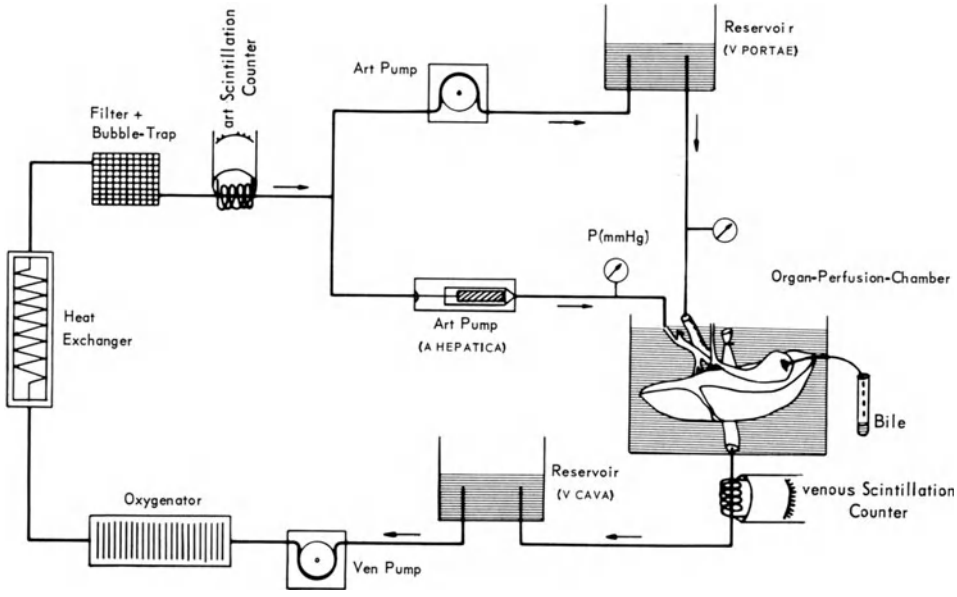

FIG. 3. Arrangement and localization of glass spirals and scintillation counter heads in the perfusion system.

As a measure of the phagocytic activity the phagocytosis index K was used, which is calculated from the particle concentration C_1 existing at the time t_1 and from the particle concentration C_2 at a later time t_2. If a quantity of particles is introduced into the circulation which exceeds the fixative capacity of the RES cells, and if the colloid concentration in the hepatic vein is not zero, then it is dependent on the function of the RES and the number of particles. Formula (a) remains valid but K is also dependent on the functional efficiency of the RES and the dose. Thus K is independent of initial concentration of particles from the liver flow and is a direct measure of the phagocytosis activity. For the calculation of K, the following formula is used:

$$K = \frac{\ln C_2 - \ln C_1}{t}$$

Therefore, on the assumption that both the activity measured on the arterial side (C_{pre}) and that measured at the end of a circulation on the venous side (C_{post}) are representative of the whole system, K is estimated as follows:

$$K = \frac{\ln C_{pre} - \ln C_{post}}{\text{Circulation time}}$$

RESULTS

A diagram comparing the time taken for the measurements with both the hemoglobin-free perfusion and the blood dilution shows that, in principle, the same time is taken by both sets of measurements of which the 10^3 counts/ml volume were plotted against time. This is based on the average values of all the measurements in the above demonstration (Fig. 4). Thus the two sets of measurements are comparable, particularly as the individual values contained in the averages of both measurements were made on the same liver in each case. In contrast to the left upper diagram, it is to be emphasized that, regarding the erythrocytic perfusion (right), the thorough mixing of the text substance and the perfusion medium persists longer here because of the reduction in flow necessitated by the increased pressure in the supply system. In the lower diagram, in which the difference between the pre- and post-hepatic average values for both perfusion media are plotted as a function of time, it is clearly seen that there is no basic difference between the two curves. From the particle concentration measured at the pre-hepatic measuring head at a time t, an approximately equal percentage is eliminated in both measurements. A difference in the phagocytic activity is only perceptible in relation to the various liver flow rates. For this reason a mean value was obtained for

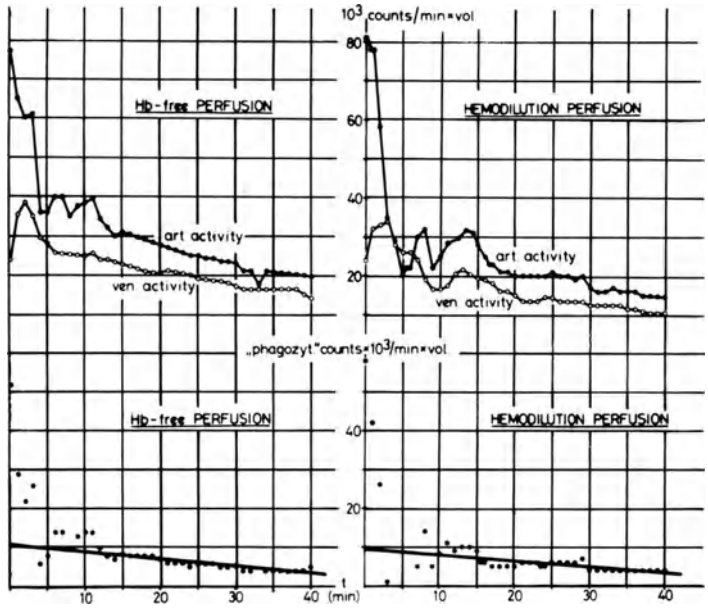

FIG. 4. Measurements of the phagocytic acitvity during perfusion with Hb-free solutions (left) and hemodilution (right).
Upper curves: Arterial and venous activity (counts/ml x volume plotted against time).
Lower curves: Difference of the pre- and post-hepatic activity measurable phagocytosis (phagocytic counts x 10^3min x vol). Pig livers were employed in these studies.

TABLE I

A COMBINATION OF TOTAL FLOW, PHAGOCYTIC INDEX K AND BIOLOGICAL HALF LIFE $t_{\frac{1}{2}}$ DURING HEMOGLOBIN-FREE LIVER PERFUSION

Number of Experiments	Total Liver Flow ml/min	K	t/2 min
1/1	400	0.0383	25
2/1	600	0.0313	23
3/1	475	0.0202	34
4/1	750	0.0568	12
4/2+	400	0.0327	21
5/1	350	0.0403	17
6/1	475	0.0230	30
6/2+	875	0.0271	26
7/1	1300	0.0340	20
8/1	450	0.0285	25
9/1	450	0.0437	16
10/1	525	0.0251	28

Data presented in Tables I-V were derived from studies on pig livers.

TABLE II

A COMBINATION OF TOTAL FLOW, PHAGOCYTIC INDEX K AND BIOLOGICAL HALF LIFE t/2 DURING PERFUSION WITH BLOOD (Hct 20-25%)

Number of Experiments	Total Liver Flow ml/min	K	t/2 min
1/2	150	0.0244	28
2/2	400	0.0183	36
3/2	200	0.0137	50
4/3	350	0.0158	44
5/2	350	0.0159	43
6/3	137	0.0126	55
7/2	350	0.0114	61
8/2	450	0.0223	31
9/2	225	0.0233	30
10/2	250	0.0106	65

the first 40 minutes after injection, since at this time even with the erythrocytic perfusion, conditions have become constant, and on the other hand a certain measurable phagocytosis has occurred. A combination of total flow, phagocytic index K, and biological half-life t/2 for the hemoglobin-free perfusion is shown in Table 1 ($t/2 = 0.693/K$).

The measurements 4/2 and 6/2 are duplicate measures with retention of the hemoglobin-free perfusion showing that the phagocytic index for the measurements are of the same order as the rest of the measurements. We may therefore conclude from the first measurements that the RES is not blocked by the test particles and a second measurement is permissible (Table 2). The same sort of combination for the erythrocytic perfusion medium shows that here also a measurable phagocytosis occurs in spite of the hypothermia. The 40 minute mean of the phagocytic index for hemoglobin-containing perfusion solutions in the same liver shows that phagocytic activity after perfusion with blood (hematocrit ca. 20 - 25) is decreased by about half (Table 3).

TABLE III

PHAGOCYTIC INDEX K 40 MINUTES AFTER PERFUSION WITH HB - FREE SOLUTION AND BLOOD

Number of Experiments	K Hb - free	K Blood
1	0.0383	0.0244
2	0.03i3	0.0183
3	0.0202	0.0137
4	0.0568	0.0158
5	0.0403	0.0159
6	0.0230	0.0126
7	0.0340	0.0114
8	0.0285	0.0223
9	0.0437	0.0233
10	0.0251	0.0106

TABLE IV

MEAN VALUES OF TOTAL LIVER FLOW, PHAGOCYTIC ACTIVITY, PHAGOCYTIC INDEX K AND HALF LIFE t/2 DURING PERFUSION WITH HB-FREE SOLUTION AND BLOOD (n=10)

Mean value	total liver flow ml/min	phagocytic activity 40'	K	t/2 min
Hb-free	590 ± 81	24 ± 3	0.033 ± 0.003	23 ± 2
Blood	286 ± 34	24 ± 5	0.017 ± 0.0016	44 ± 4
p	<0.01		<0.001	<0.001

This is particularly clearly shown for all quantities by the mean values (Table 4).

It emerges quite clearly from this that the difference of the (pre- and post-hepatic) activity (in percent) is the same in both hemoglobin-free and hemoglobin-containing perfusions. The total flow of the Hb-containing perfusion solution is about 52% lower than that of Hb-free solution. In spite of the same percentage difference of the measurements for the Au^{198} concentration in both series of tests, the phagocytic activity is reduced by about 49% compared with the initial value, due to changes in flow.

Since a relationship between the duration of the perfusion until measurement and the phagocytic activity cannot be established (Table 5), it follows that the functional efficiency of the RES with prolonged perfusion with hypothermia is not impaired by Hb-free solutions.

TABLE V

DURATION OF PERFUSION (TIME = h) COMPARED WITH THE PHAGOCYTIC INDEX K

Time of perfusion (h) until measurement	K
1.5	0.0313
3.5	0.0202
4.5	0.0285
5.5	0.0568
6.0	0.0403
6.5	0.0230
7.0	0.0340
10.5	0.0271
12.0	0.0251
12.5	0.0437
20.0	0.0383
23.0	0.0327

TABLE VI

COMPARISON OF THE MEAN VALUES OF TOTAL LIVER FLOW AND PHAGOCYTIC INDEX K OF PIG, DOG AND HUMAN LIVERS DURING PERFUSION WITH HB-FREE SOLUTION (HYPOTHERMIA 10°C) AND BLOOD

Pigs (n = 10)		
Hb-free solution	590 ± 81	0.033 ± 0.003
Blood (Hct 20-25%)	286 ± 34	0.017 ± 0.0016
Dogs (n = 6)		
Hb-free solution	340 ± 41	0.0306 ± 0.002
Blood (Hct 20-25%)	196 ± 56	0.0178 ± 0.0012
Men (n = 3)		
Hb-free solution	550 ± 30	0.0955 ± 0.003
Blood (Hct 20-25%)	450 ± 22	0.0575 ± 0.002

Results obtained from the pig and dog livers compared with the measurements in human liver show that the phagocytic activity of the livers of different laboratory animal species is essentially the same as that of perfused human liver under the conditions applied in our experiments (Table 6).

Investigation of the ultrastructures of the sinusoidal cavities in perfusion with heterologous blood already shows after 3 to 6 hours that the sinusoidal endothelium folds up into a labyrinthine form, while Disse's space is enlarged and is filled with coagulated protein. The liver cells show normal mitochondria and glycogen content. After 6 hours of perfusion the lumen of the sinusoid in certain areas is completely filled with swollen phagocyting cells. Increased blood flow is found and usually blood cells are observed in Disse's space, both due to the insufficiency of the sinusoidal endothelium. The reduction or increase of insufficiency of this barrier seems to run parallel to the measurable changes in the function of the liver.

In summary, the following points can be set out:

1. The parameters obtained for phagocytosis by Hb-free and Hb-containing perfusion solutions are comparable.

2. There is no significant influence on the RES dependent on time in perfusion with Hb-free solutions.

3. The phagocytic activity with Hb-containing perfusions is significantly reduced compared to that with Hb-free solutions.

4. From individual investigations it is shown that the reticuloendothelial system in the livers of various animal species, particularly in man, behave similarly in isolated liver perfusion with hypothermia.

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FUNCTION STUDIES OF THE SPLEEN USING RADIOACTIVE-LABELED AND HEAT-DENATURED ERYTHROCYTES AND THEIR CLINICAL VALUE

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The RES as an especially active, functionally versatile, and very extended system has its most important concentration of tissue in the spleen. Till now it was possible only to investigate this organ by cytological and histological methods. Clinical function tests of the spleen were completely unknown until recently. Such investigations have now become possible by labeling the blood cells with gamma-emitting radioisotopes and the development of external counting techniques.

For spleen function studies we use ^{51}Cr-labeled erythrocytes damaged by heat as they have been applied for splenic scanning since 1960. Measurement of blood activity and of the accumulation of activity in various organs can be carried out by means of special equipment developed by us, the so-called 12-channel function device "MAINZ" (1,2).

By heating erythrocytes to 49.5°C for 20 minutes a mixture of about 70 - 80% spherocytes of 5 u diameter and about 20 - 30% erythrocyte fragments are produced. The characteristics of this mixture are widely independent of the donor, so that one can always use the patient's own red blood cells. After injection of heat-damaged, ^{51}Cr-labeled erythrocytes, one observes a biexponential time course in the blood (Fig. 1). About 60 - 75% of the initial activity is eliminated with a half-life of about 4 minutes and is accumulated with the same half-life in the spleen. The rest of the activity is extracted much more slowly (half-lives of about 30 - 50 minutes) and accumulated in the liver and the bone marrow.

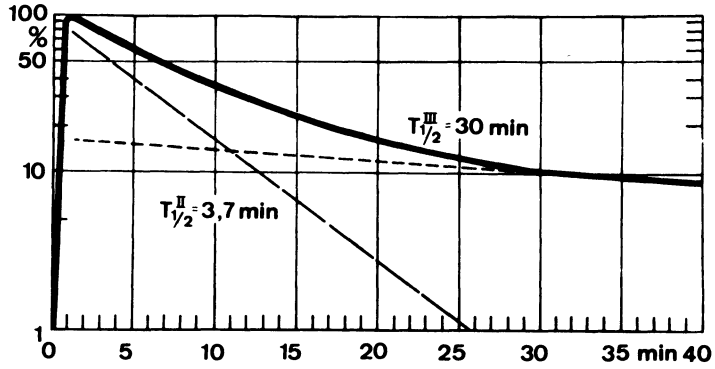


Fig. 1. Time course of activity in the blood after injection of heat-damaged erythrocytes in a normal subject. Y-axis: percentage of maximum activity on a logarithmic scale; x-axis: time on a linear scale. The course of activity can be separated into two exponential functions. 60 - 75% of the maximum initial activity is eliminated with a half-life of minutes.

From these facts one can conclude that the spherocytes are selectively sequestrated by the spleen and the fragments are phagocytized by the RES. The assumption that the rapid component of the extraction is caused by splenic trapping is confirmed by the observation that in splenectomized subjects this rapidly eliminated component is absent (Fig. 2). Thus with the heat-induced spherocytes a substance is at hand with which it is possible to investigate clinically a certain specific behavior of the spleen which is characterized as follows.

The normal spleen is able to sequestrate a rapidly injected amount of about 3 - 5 ml of heat-damaged red blood cells with high efficiency. By injection of heat-denatured erythrocytes into the splenic artery intra operationem we were able to demonstrate that the degree of efficiency for the extraction of spherocytes is about 100% in normals, so that the velocity of extraction in such cases is only regulated by splenic blood flow. In connection with this two questions arise:

1. What is the recognition mechanism of the spleen for these cells?

2. What part of the spleen is responsible for the sequestration of spherocytes?

Heating of erythrocytes induces a complete loss in an essential feature of the normal red blood cells, namely, its plasticity

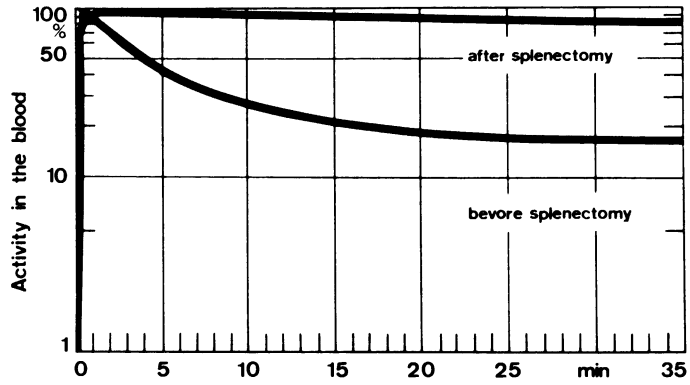


Fig. 2. Time course of activity in the blood after injection of heat-damaged erythrocytes in a splenectomized subject. The decrease in activity is monoexponential; the rapid component is absent.

or fluidity (3). The fluidity enables the erythrocytes to pass through apertures which are smaller than their own diameter. These smallest apertures in the circulation are located in the red pulp of the spleen. The heat-induced spherocytes are rigid and therefore unable to pass these splenic cords and are retained there. The totality of the splenic cords can therefore be regarded as one uniform filtration space. In contrast to the red pulp, the white pulp never participates in the extraction process. If the filtration space is reduced beyond a certain extent this fact must be reflected in the result of the function test.

Such a reduction of the filtration space of the spleen can be caused by:

1. Proliferation of the white pulp (e. g., ITP, giant follicular lymphoblastomas)

2. Autonomous proliferation of the reticulum of the red pulp

3. Reactive proliferation of the reticulum (e. g., inflammations of diverse origin, metabolic diseases)

4. Obstructions of the filtration space (e. g., primary polycythemia, secondary polycythemia, hemolytic anemias)

5. Widespread parenchymal lesions (e. g., infarctions, abscesses)

The loss of specific filtration space can be compensated to a greater or smaller extent by an enlargement of the spleen. It is therefore necessary to combine the functional investigation with a quantitative determination of spleen size by means of scintigraphy. We have applied this combined investigation in more than 5000 cases. Before demonstrating the results by some examples of well-defined diseases, it must be pointed out that the function test using heat-denatured erythrocytes must not be confused with the measurement of erythrocyte lifespan. The determination of red blood cell survival is concerned with the question how long the erythrocytes of the patient remain within his circulation, while the former method is related to the question whether or not the spleen of the patient is able to sequestrate a small amount of heat-denatured red blood cells within a short time, as defined by the normal range.

Figure 3 shows the result of the test in 44 patients with acute leukemia before treatment. The coordinates indicate the weight of the spleen as determined by scintigraphy on the x-axis and the so-called sequestration index, i. e., that part of the initial activity in the blood which is eliminated rapidly, on the x-axis. The column indicates the normal range for the weight (80 to 200 g) and the horizontal lines represent the normal range for the sequestration index (60 - 75%). Only two of the 44 cases are within the limits of normal values.

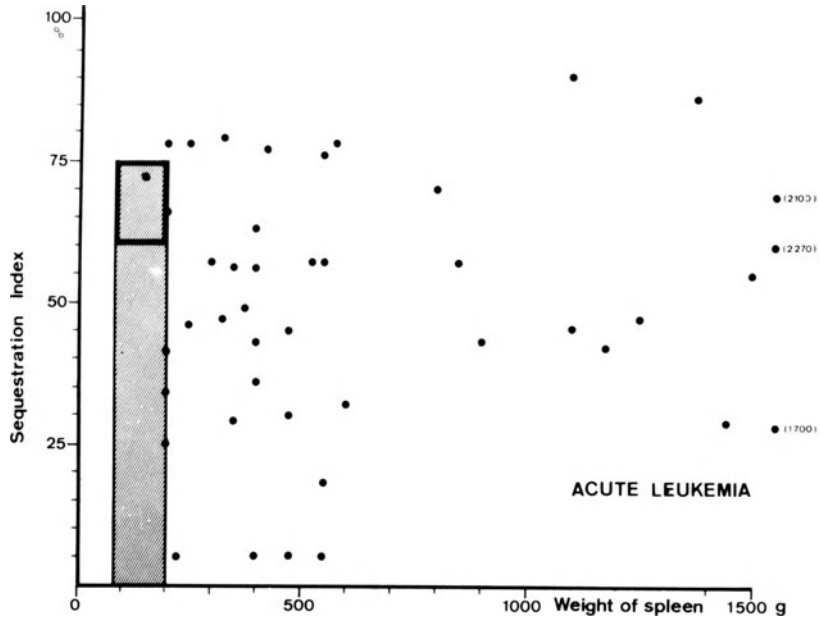


Fig. 3. Results of the combined functional and scintigraphical investigation of the spleen in 44 cases of acute leukemia.

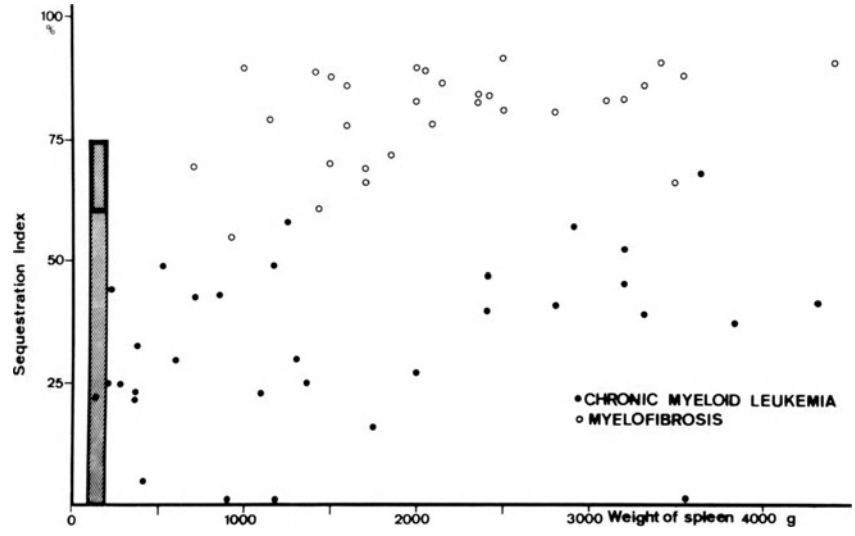


Fig. 4. Size and function of the spleen in 32 cases of myeloid leukemia (black points) and 31 cases of myelofibrosis.

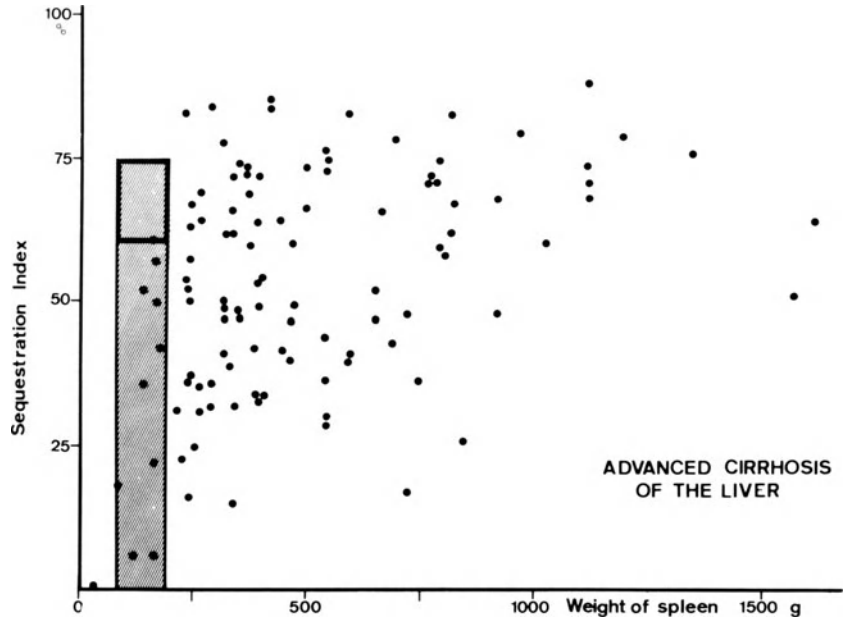


Fig. 5. Results of function test in 112 histologically proved cases of advanced cirrhosis of the liver.

Figure 4 shows the behavior of size and function of the spleen in 32 cases of chronic myeloid leukemia and 31 cases of myelofibrosis. One can see that in the case of leukemia (black points) even an extremely large spleen is not able to sequestrate a relatively small amount of injected heat-denatured erythrocytes. In contrast to this, in all cases of myelofibrosis there is a normal uptake of these cells. This is an example for the case that the loss in filtration space is compensated by the enlargement of the organ. The results of the function test can serve as a differential diagnostic aid between myelofibrosis and chronic myeloid leukemia.

In Figure 5 results are demonstrated in 112 histologically proved cases of advanced cirrhosis of the liver. Only one case is within the normal range. Though there is a considerable enlargement of the spleen in many cases the sequestration index is low, indicating that there are pronounced histological modifications of the splenic tissue which lead to a reduction of the filtration space.

Figure 6 demonstrates the results of the investigation in 108 patients suffering from carcinomas. In this group, in which one does not consider an involvement of the spleen in the disease, we found that the sequestration capacity often was reduced.

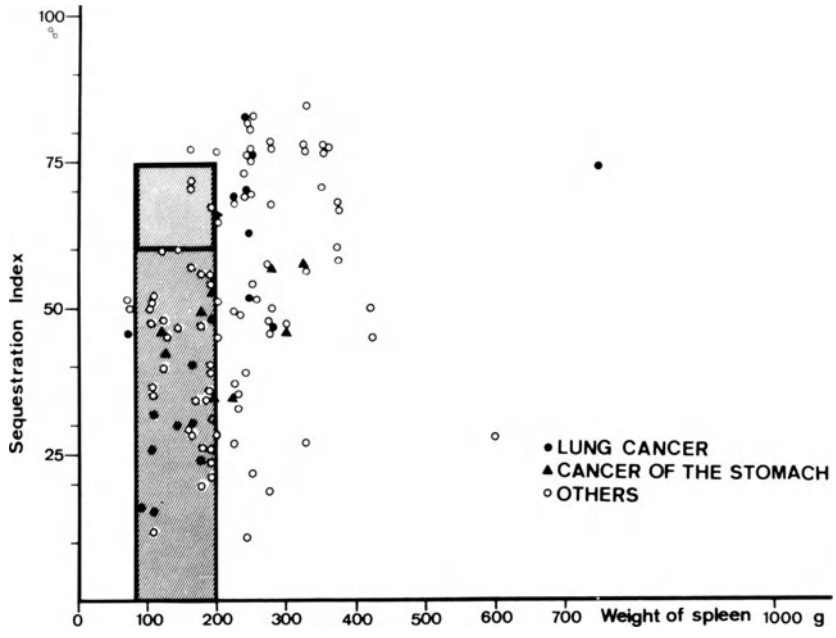


Fig. 6. Results of function test in 108 patients with carcinoma.

Size and Functional Condition of the Spleen
Chronic Myeloid Leukemia
Continual Treatment with Cytostatics

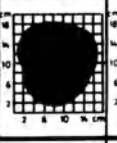
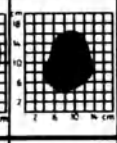
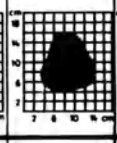
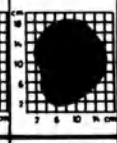
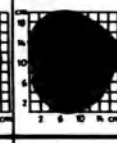
Date	18 2 1963	14 3 1963	14 5 1963	9 8 1963	25 9 1963	19 2 1964
Leucocytes (per mm³)	23 000	12000	10700	12 500	95000	260000
Pathological Forms	29 %	2 %	-	12 %	66 %	70 %
						
Surface Area in the Scintigram	255 cm²	210 cm²	90 cm²	95 cm²	190 cm²	280 cm²
Weight of Spleen	1750 g	1250 g	250 g	275 g	1100 g	2 100
$T_{1/2}$ of Heat Altered Erythrocytes Clearance	22 min	11 min	11 min	39 min	80 min	32 min
Sequestration in the Spleen	16 %	54 %	62 %	8 %	4 %	17 %

Fig. 7. Results of the functional and scintigraphical investigation of the spleen in a patient with chronic myeloid leukemia during treatment.

Finally, we would like to point out the clinical importance of the combined functional and scintigraphical investigation of the spleen for the observation of the course of a disease. Figure 7 demonstrates that at the beginning of the investigation a large spleen (1500 g) can sequestrate only 16% of the injected amount of heat-damaged erythrocytes. After treatment which led to a reduction of the spleen weight to 250 g the sequestration is normal. The next column indicates that the sequestration index fell to 8% though the weight of the spleen was nearly unchanged. At this moment no pronounced hematological disorders could be observed. Then a considerable enlargement of the spleen occurred, which was accompanied by a further reduction of the sequestration index. Though therapy was continued, no amelioration of the disease was possible. In this case the whole reticular network of the spleen - as shown on autopsy - was completely transformed by proliferation of myelofibrosis. Practically no filtration space was left.

The functional test described here enables us to evaluate clinically and quantitatively the behavior of the RES within the spleen. The results of our investigations demonstrate how often the RES of this organ is affected in various diseases. The information value of this investigation is therefore comparable to that of the red cell sedimentation rate.

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THE EVALUATION OF PHAGOCYTIC ACTIVITY IN MAN BY MEANS OF A LIPID CLEARANCE TEST

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In a variety of diseases the role of the reticuloendothelial system is of central importance. Depending on the causing factors, its manifold functions may be stimulated or depressed, respectively. The activity of the RES is for instance raised during pregnancy, in rheumatic and allergic diseases, after bacterial infections and in the early stages of malignant diseases. A reduction in activity occurs with old age, in diabetes mellitus, in leukemia, in gram-negative and viral infections, after severe injuries, and in other conditions producing shock. The functions of the RES can also be blocked by drugs, such as cytostatics, corticosteroids, colloidal substances, various antibiotics and anesthetics.

In animal experiments, tests have been made of substances which stimulate the RES, such as estrogens, BCG and pertussis vaccines, endotoxin in small doses, zymosan, glucan and triolein, as well as restim (1,2,3), a lipid fraction from shark liver, and aristolochia acid, a plant extraction. As most of these agents cause a hyperplasia of the RES, e.g. a granuloma formation in liver, spleen and lungs, they have not been used yet to increase immunity in humans; with the exception of BCG (4).

In order to get an insight into the patients resistance, one parameter of reticuloendothelial function, the phagocytosis of colloids from the blood stream, was examined. There appears to exist a close relationship to other cellular activities such as antibody formation and detoxification. Colloidal particles, when intravenously injected, are phagocytized by the RE cells at a certain rate and the decline in serum can be measured as an

expression of phagocytic activity. After a high rate of complications with a I^{131}-marked dispersion of human albumin microaggregates, we compared three different lipid emulsions: a) safflower oil according to Salky and Di Luzio (5), b) cotton seed oil as used by Biozzi and Benacerraf (6) and soia bean oil (7), which in Germany is available for parenteral nutrition. Since the latter was phagocytized at the same rate and showed the least side effects during the test period, we decided for Lipofundin-S from Fa.B. Braun-Melsungen, Germany.

MATERIAL AND METHOD

The oil-in-water emulsion Lipofundin-S-20 consists of 20% soia oil, 1.5% soia phosphatide, 5.0% xylite, and 73.5% water. Thirty ml of the emulsion per squaremeter body surface, e.g. approximately 40 to 70 ml Lipofundin-S-20 per patient are injected exactly within 2 min and blood samples of 2 ml (including 0.4 ml citrate) are taken after 2, 4, 6, 8 and 10 minutes. After dilution with 4ml saline, the samples are centrifuged for 2 min at 1000 G, the ascended fat is stirred back into the serum, and turbidity is measured spectrophotometrically at a wave length of 580 nm. Half-life of the lipid is then calculated on a semilogarithmic chart.

The dose of lipids had to be taken as high as approximately 150 mg/kg bodyweight in order to exceed the "critical dose", when T/2 is a true expression of phagocytic activity and not of liver blood flow. The Lipofundin emulsion with a particle size of 0.1 to 0.5μ in diameter was relatively stable after the injection since blood samples of test persons as well as histological sections of liver and lung revealed no particles larger than 2.0 μ in diameter. The injected lipids were rapidly metabolized and most of the RE cells were already emptied 15 min later when the parenchymal cells of the liver took over the lipid containing vesicles. There was no detectable intravascular lipolysis, since blood levels of free fatty acids did not rise significantly during and after injection. Lung sections taken 5 and 15 min after the injection of Lipofundin-S revealed no signs of fat embolism. Despite this finding, tests were never performed in patients with volume deficiency, low blood pressure, electrolyte disturbances or acidosis.

RESULTS

Four hundred fifty patients tolerated the test without remarkable complications. Eight patients, so far, e.g. 2%, had early symptoms of a so-called"colloid shock" with heat sensation,

dyspnea, nausea or shivers. Two of these patients were tested 4 and 6 times before and probably developed signs of an allergic reaction. Other patients and healthy students tolerated the test up to 4 times a day without any discomfort. For inexplicable reasons, the first 56 control persons had mean T/2-values of 2.8± 0.5 which were confirmed in the same persons by additional tests with cotton seed oil and safflower oil.

The mean values of the clearance rates in groups of at least 10 patients are compared in Figure 1. In 106 healthy control patients, the half life of Lipofundin-S-20 ranged between 3.1 and 7.4 min with a mean value of 5.0± 1.2 min. So far, this value appears to be independent of age and sex. All half-life periods below 3.0 point to a stimulation of RE functions, f.i. by bacterial infections or tumor growth; all half-life periods over 7.5 min are a sign for RE depression, as if is obvious in patients after severe injury with shock, or in patients having had narcosis and surgery.

In patients going through a period of traumatic shock, clearance values up to 18.3 min were measured 58 hours after the accident. This is an expression of a 3-fold decrease in phagocytic activity, probably with a consequent impairment of antibody - formation, detoxification, lipid metabolism and conjugation of steroids. In this view, the development of a stress ulcer or

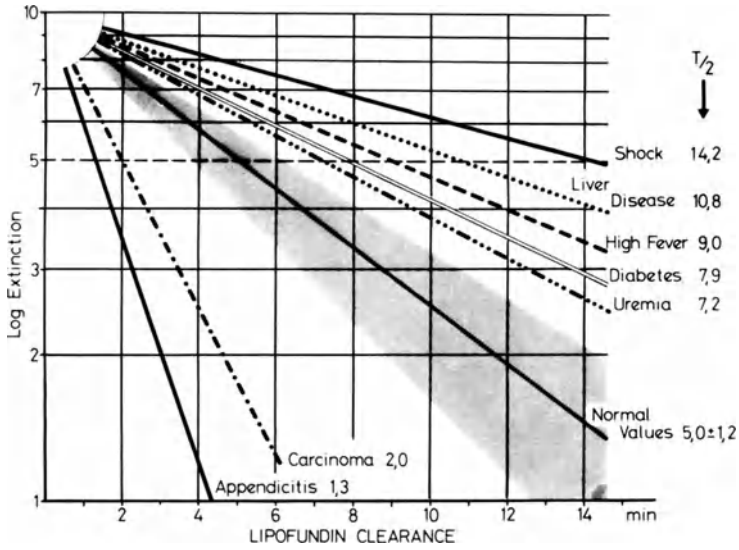

Figure 1: Clearance rates of patients with different diseases. Each value represents the mean of at least 10 patients.

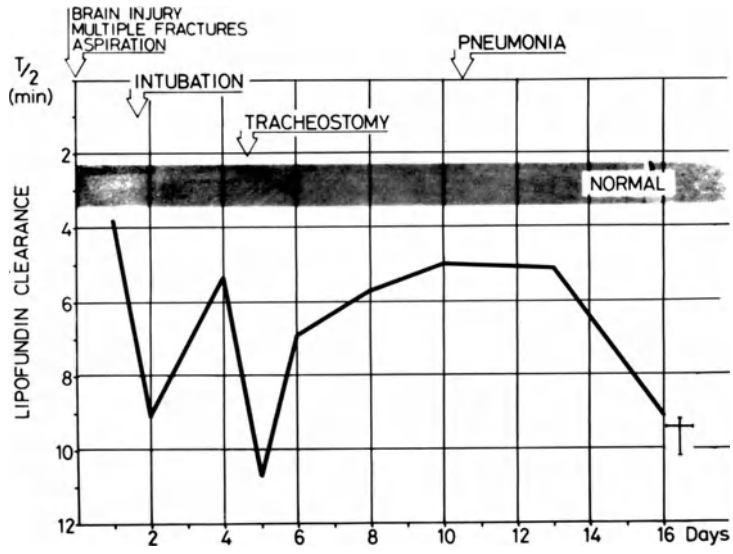

Figure 2: 18-year old male, car accident, artificial respiration death from pneumonia in day 16.

fat embolism after a period of delayed shock becomes intelligible, as well as the diminished resistance towards infections. Furthermore, patients with uremia, liver cirrhosis, and respiratory or cardiac diseases with severe hypoxia had significantly prolonged clearance rates (Fig. 1).

In various patients with multiple fractures or severe burns (Fig. 3), a clear dependence of RE function on blood oxygen pressure was obvious. In shock, a certain blood volume is stored in the mesenteric blood vessels with reduction of liver blood flow. Consequently, hypoxia causes a swelling of the endothelial cells lining the liver sinus, which again may lead to an increased portal venous pressure

On the other hand, a variety of drugs has been shown to depress phagocytic activity in mice and rats (8). If one looks at an intensive care unit where each patient receives at least 10 different drugs a day, one is surprised that they acquire pneumonia despite high antibiotic prophylaxis (Fig. 2). The RES of surgical patients has to process a heavy load of cell debris, fibrin clots, toxins and liberated enzymes, which can lead to a temporary blockade. According to our experiences in rodents, each contact with gram-positive bacteria leads in healthy subjects to an activation of immune mechanisms, whereas an already damaged

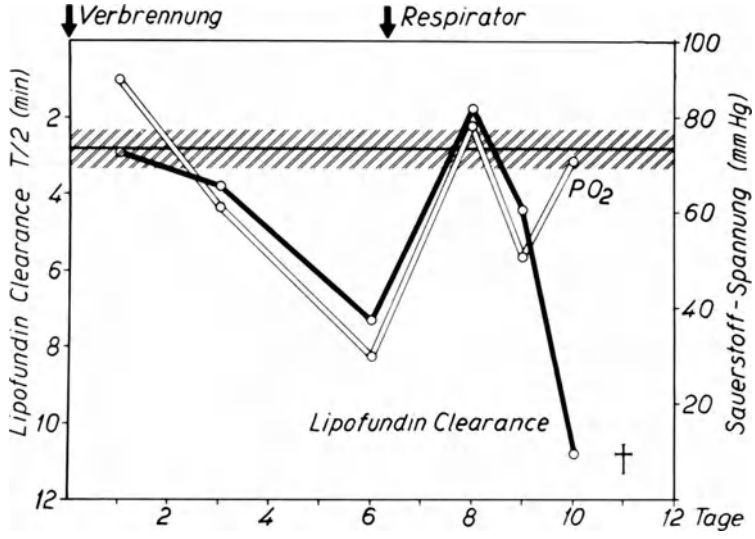

Figure 3: 26-year old male, third degree burn of 55% of the body surface. Death on day 11 from pneumonia.

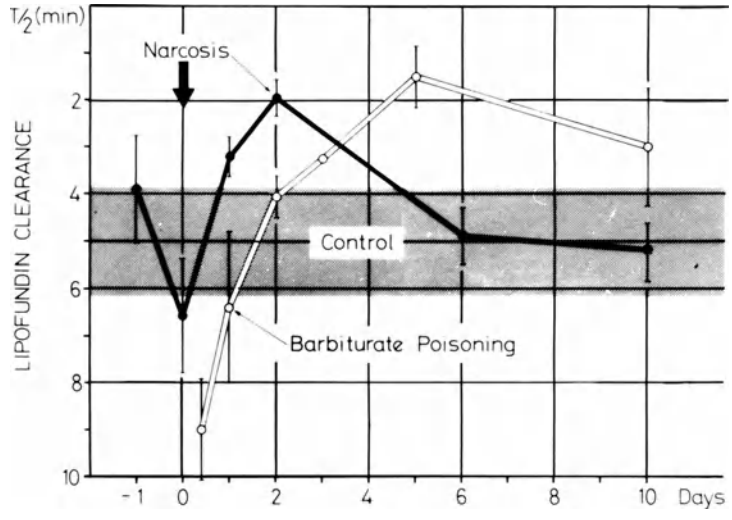

Figure 4: Lipofundin clearance rates in patients which underwent narcosis or took an overdose of sleeping pills.

RES may succumb to the least additional burden.

Patients who were tested the day before and during narcosis (Fig. 4) showed a slight depression of phagocytic activity caused by the anesthetics. Three patients with herniotomia under local anesthesia had no obvious impairment of RE functions, but the same slight stimulation during the following days. The depression under fluothane narcosis lasted usually for 6 hours and was dose and time dependent. Experiments in mice stated clearly, that fluothane and barbiturates were responsible for RE depression while ether and nitrogen oxide had no effect on phagocytosis. Therefore, in severely injured patients, one should postpone the indication for an operative treatment until RE function has fully recovered and is able to overcome a new impairment.

In our experience, concussion of the brain or severe brain damage had little influence on the activity of the RES (Fig. 5). The reported results of the Lipofundin-Clearance-Test are preliminary and because of many uncontrolled factors in clinical treatment, not absolutely evident. They confirm the measurements of other studies (9) and show, that this test might be a simple and useful tool in proving the effectiveness of certain therapeutic efforts and hopefully of RE stimulating agents for the clinical use.

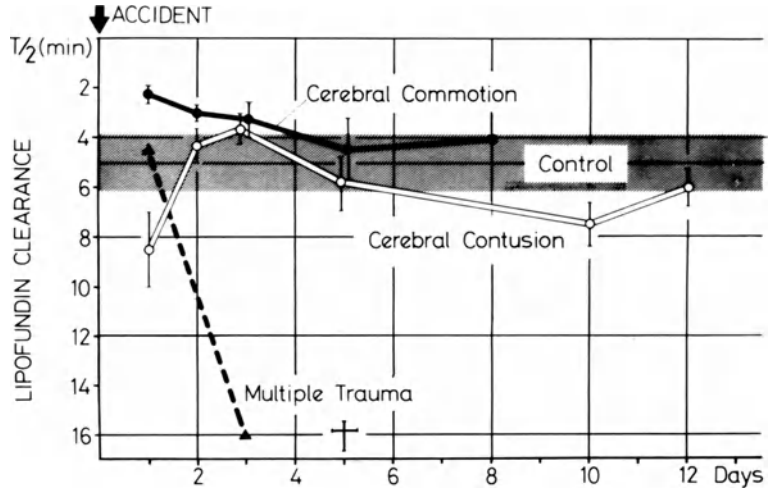

Figure 5: Phagocytic activity is not altered significantly in patients with severe brain damage or even brain death.

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POSSIBLE PARTICIPATION OF SERUM-COMPLEMENT IN THE INTRACELLULAR KILLING OF E. COLI [+]

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Complement (C) was detected in 1889 by its bactericidal activity against gram-negative bacteria (1). In 1903 (2), it was discovered that C also had an opsonizing effect on bacteria, even if they were resistant to the bactericidal activity. Optimal opsonization was obtained when the third component of C was activated and attached to the bacterial surface (3) whereas for the bactericidal activity all nine components were required (4,5,6).

The majority of pathogenic bacteria is now known to be resistant to the bactericidal activity of C. They are readily opsonized and killed intracellularly following phagocytosis. If the activity of C to the C-resistant bacteria were limited to opsonization by the activation of its third component, it would mean that in this case an individuum would not make use of all following components, i.e. C5 - C9, when defending against such infections.

The experiments of Li et al. (7) suggested that this may not be true. The authors compared the phagocytosis of bacteria treated with normal and heated serum. They found poor phagocytosis and no intracellular killing when heated (56°C, 30 min.) serum was used for opsonization, but good phagocytosis and rapid intracellular killing when normal serum was used. The authors concluded that C must be important for phagocytosis as well as for intracellular killing. Support

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for the conclusion came from experiments of Jenkin (8). Working with lysogenic phages, phage antibodies and C, he showed that bacteria multiplied within the phagocytes when phagocytosis was due to the opsonized phages adhering to the bacterial surface. However, when bacteria were opsonized with bacteria-antibodies and C, they were readily killed within the phagocytes.

In contrast Craig and Suter (9) could not detect any difference in the intracellular fate of bacteria treated with normal, heated, EDTA-treated or Zymosan-treated serum.

This communication reports a new approach to this problem. The intracellular killing of bacteria by PMNs was observed. C-resistant E. coli were either treated with the opsonizing components of C alone or with all nine components of C. It will be shown that the late reacting components of C augment the intracellular degrading process.

MATERIALS AND METHODS

Tissue Culture Tubes, Leyton type, were used with two cover slips 9 x 22 mm.

Medium 199 (Colorado Serum Comp.) was adjusted to pH 7.2 and was used in all experiments.

Polymorphonuclear leucocytes (PMN) were harvested from pig peripheral blood. Heparinized (5 I.U./ml) blood was diluted with dextran (2 vol. blood to 1 vol dextran) (Macrodex, Knoll AG, Ludwigshafen, Germany) to a final concentration of 3 % dextran. After one hour the supernatant was removed and centrifuged 5 min. at 1200 rpm. The white blood cells were resuspended in Medium 199 and adjusted to 10 x 10^6 PMN/ml. Of the PMN-suspension 2 ml were incubated in tissue culture tubes for 1 h at 37^o following which the PMNs had formed a monolayer on the cover slips. Cells that were not fixed on the cover slip were rinsed off. The monolayer consisted of 84 - 87 % PMNs.

Bacteria: C resistant E. coli E56b with the serotype O8:K27-H were kindly supplied by Dr. Schlecht and Dr. Schmidt of our institute. The bacteria were used in the log-phase after 3 h of cultivation in brain-heart-infusion (Difco) at 37^o.

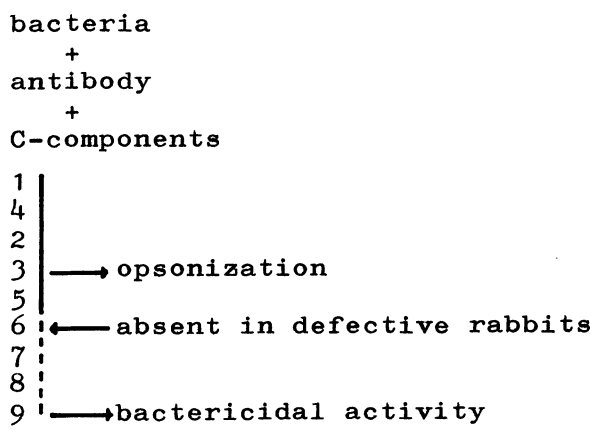
Antibodies (Ab) were raised in rabbits. Formalin inactivated E. coli were suspended in saline to a concentration of 10^9 bacteria/ml. Rabbits were injected i.v. at day 0 with 0.25 ml, at day 5 with 0.5 ml and

at day 10 with 1.0 ml bacterial suspension. The animals were bled on day 15. The Ab-titer was measured by agglutination. The limit of agglutination was at a serum dilution 1:800 to 1:3.200.

Complement: Whole C was used from a pool of normal, C-active rabbits. Defective C (C def.) was used from a pool of sera from C6 deficient rabbits. The animals lacked by heredity the 6th component of the C sequence, the other components were present. Since the sequence was interrupted at the C6-step, opsonization could occur, but the components C7 - C9 were not activated as shown in Table I.

Opsonization: The bacteria were sensitized 20 min. at 37° and opsonized 10 min at 37° with a) normal C, or b) defective C.

Table I



Phagocytosis: The monolayer of PMNs was inoculated with 1 ml opsonized bacteria (s. results). Following time intervals (s. results) the cover-slips were removed and stained by Pappenheim's stain. The number of PMNs that contained ingested bacteria was counted microscopically as a measure for phagocytosis.

Intracellular killing: After a time period (s.results) of ingestion the phagocytosis was stopped by rinsing off all bacteria that had not been phagocytized. Tubes were washed 3 times with saline of 37°. One sample was examined immediately after separation of phagocytized and not phagocytized bacteria. The two

cover slips were removed from the tubes. One cover slip was stained and used for microscopical examination. The second cover slip was used for measuring the count of viable bacteria within the PMNs. The procedure was this: To stop further intracellular process and to lyse PMNs, the cover slip was put into ice cold, sterile tap water. Bacteria were set free by vigorous shaking. The amount of viable bacteria was determined by colony counting on agar after 18 h incubation at 37°. The number of viable bacteria at the end of phagocytosis was taken as the reference point (100 %).

The other tubes were reincubated at 37° immediately after nonphagocytized bacteria had been separated, and samples were withdrawn at intervals and examined as described above.

RESULTS

Phagocytosis

Preliminary phagocytosis experiments were performed to determine optimal conditions for the intracellular killing experiments. The latter should be observed in the absence of continued phagocytosis. To examine the fate of ingested bacteria, it was necessary to keep the time period of phagocytosis short when compared to the period of intracellular killing.

Figure 1 shows the influence of four parameters on the phagocytosis of bacteria: (a) The ratio number of bacteria added to number of leucocytes present, (b) the concentration of Ab, (c) concentration of C, (d) the duration of phagocytosis. It can be seen that phagocytosis increases with the increase of the ratio of number of bacteria added/number of leucocytes, with the degree of sensitization, the degree of opsonization and the time of phagocytosis. Five min. of phagocytosis were sufficient to exceed the phagocytic rate of 50 %. The time of phagocytosis in the intracellular killing experiments was therefore limited to 5 min. The inoculum of bacteria was 3 x 10^8/tube. This gave maximal phagocytosis within 5 min. of phagocytosis. Ab was used in 1 - 1/2 agglutination units. The C-containing serum was diluted 1:20 - 1/40.

The next experiment was designed to exclude a possible difference in the phagocytosis in the two

series. Intracellular killing could be compared only when equal amounts of bacteria had been phagocytized. As demonstrated in Table II, there was no difference between the bacteria opsonized with C and those opsonized with C def. The phagocytosis of bacteria treated with C or C def alone was relatively high with 14 % and 19 % respectively, possibly due to natural antibodies present in the C and C def-containing serum. Since the bacteria in the two experiments were similarly opsonized, the influence of the natural antibodies could be neglected.

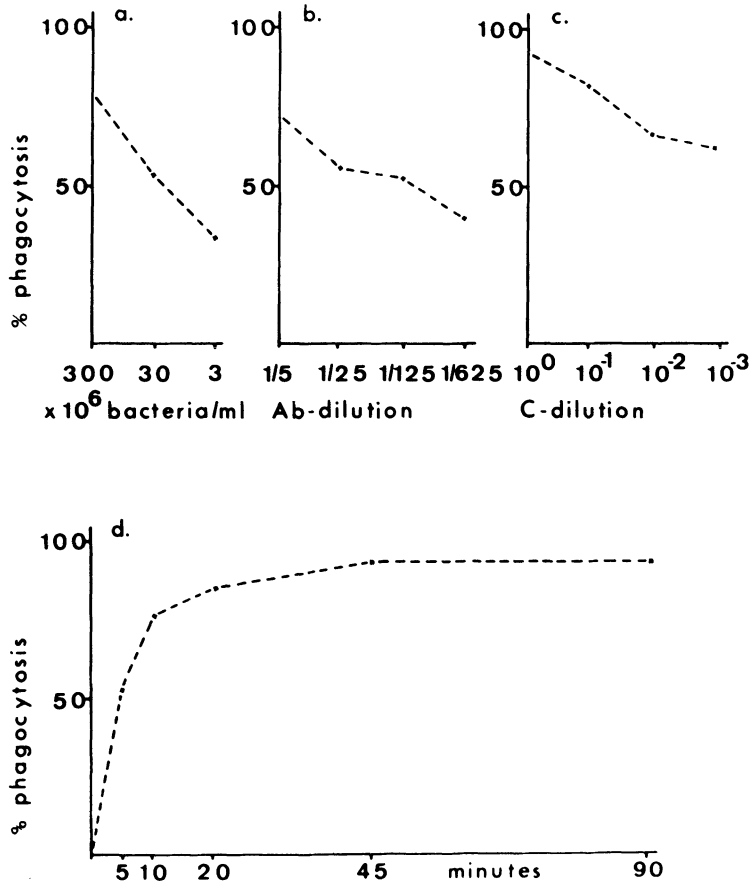


Fig. 1. Influence of four parameters on phagocytosis: (a) ratio of number of bacteria added over number of PMNs present (monolayer, (b) Ab-dilution, (c) C-dilution, (d) time of phagocytosis

Table II

bacteria treated with:	% phagocytosis
NaCl	2
Ab + NaCl	16
NaCl + C	14
NaCl + Cdef	19
Ab + C	55
Ab + Cdef	57

Intracellular Killing

The rate of intracellular killing, as measured by the colony counting method, is shown in Fig. 2. Only 12 % of bacteria opsonized by C-active serum and living at the time of reincubation survived for 20 min.

In contrast, bacteria opsonized by defective serum survived longer. After 20 min 35 % were still viable. The exposure of the C-resistant E. coli to all nine C components resulted in a higher rate of intracellular killing when compared with the exposure to the first four components only.

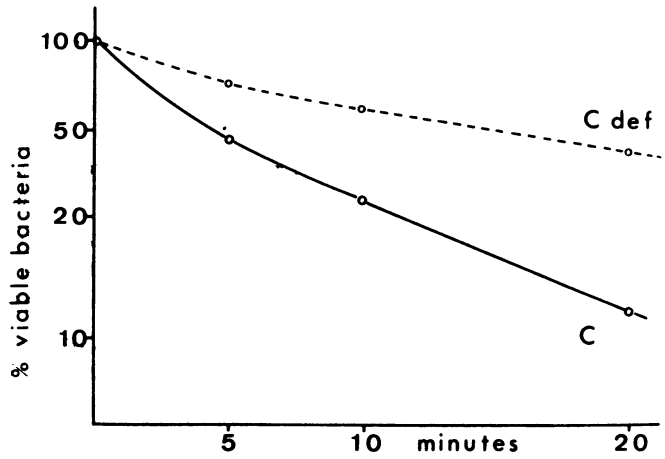


Fig. 2. Intracellular killing of E. coli 08:K⁻ by PMN measured by colony counting. Solid line: E. coli opsonized with C. Dotted line: E. coli opsonized with C def.

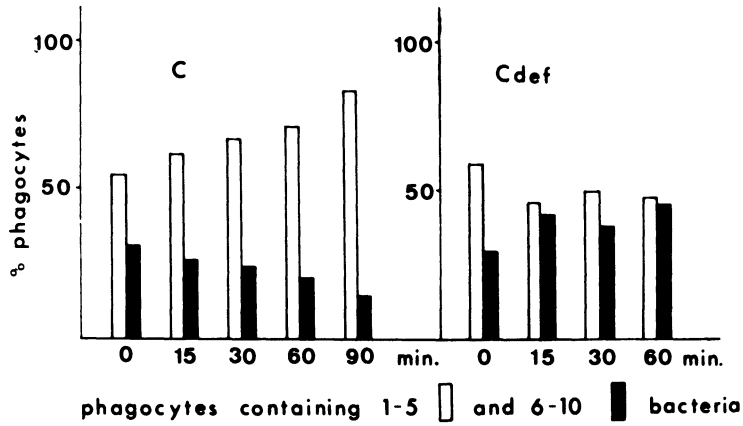


Fig. 3. Microscopic examination of intracellular degradation of E. coli by PMN. Bacteria treated with C and C def, light bars: PMN with 1 - 5 ingested bacteria; dark bars: PMN with 6 - 10 ingested bacteria.

We then tried to establish the result by another method. When the cover slips were examined microscopically a similar difference in the 2 series was found in the intracellular degradation as demonstrated for intracellular killing by the colony counting method.

The phagocytes were divided in several groups, one containing 1 - 5, the next 6 - 10 bacteria/cell groups of phagocytes containing more than 10 bacteria are not depicted here. They do not alter the result. The portion of each group among the total number of phagocytes at different time intervals is demonstrated in Fig. 3. When normal C was used for opsonization the portion of cells containing 1 - 5 bacteria increased with time of incubation, whereas the portion of cells containing 6 - 10 bacteria decreases. In the parallel experiment, when C def was used for opsonization, this shift was not seen.

It was concluded from these data that one or more of the late reacting components of C, i.e. C5 - C9, enhanced the effectivity of intracellular killing and degradation.

DISCUSSION

Phagocytes were able to kill and digest bacteria which had not been exposed to the components 6 through 9 of the C system prior to ingestion. Pretreatment with all C components, however, enhanced intracellular killing. Since a possible continuation of an extracellular process of C bactericidal activity was excluded by the use of a C resistant strain of bacteria, the enhancing effect may be explained in two alternative ways: (1) C might attack some structures of the bacteria making them more vulnerable to the intracellular bactericidal mechanism. This would limit the action of C to the extracellular phase. (2) The activated C components attached to the cell wall and ingested with the bacteria attack phagosomal membranes in a manner analogous to the reactive lysis recently described by Thompson and Rowe (10) and by Götze and Müller-Eberhard (11). The enhanced permeability of the phagosomal membrane would then faciliate the access of bactericidal enzymes to the ingested bacteria. This interpretation would suppose a continued activity of C within the cell.

The much discussed question whether C normally participates in the intracellular killing of bacteria and if so, to which extent, has not been investigated in the present study. Since it has been shown by Thorbecke et al. (12) and Littelton et al. (13) that white blood cells especially macrophages contain and/or produce several of the C components, this question could only be answered on the basis of experiments with phagocytes of C deficient animals. In the experiments where opsonization was achieved with the C components 1 through 5 only, the presence of these factors could carry the C sequence to completion and thus contribute to the high rate of intracellular killing.

Under normal conditions the life expectancy of C6 deficient rabbits (14), of C5 deficient mice (15) and of C4 deficient guinea pigs (16) is not diminished. A recent report of Shin et al. (17), however, suggested a vital role of C in the defense of the body against infections under special conditions. They demonstrated a higher mortality of C5 deficient B10D2 old line mice as compared to C active B10D2 new line mice when challenged with high doses of pathogenic bacteria.

SUMMARY

The influence of C on the intracellular killing of ingested bacteria was investigated. C resistant E. coli were opsonized with (1) serum of C active or (2) serum of C defective rabbits. The bacteria opsonized by normal C were killed faster within PMNs than bacteria opsonized by deficient C. It was concluded that the late reacting components of C influence the intracellular fate of bacteria. The significance of this phenomenon in the framework of host defense to infections is discussed.

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FATE OF STREPTOCOCCAL ANTIGENS IN THE RETICULOENDOTHELIAL SYSTEM OF THE MOUSE LIVER

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Because of the clinical and epidemiological evidence that group A streptococci play a major role in the pathogenesis of rheumatic fever and glomerulonephritis and the belief that these may be immunologic diseases (1), a number of investigations have centered on the in vivo distribution of or the host immunologic response to various components of these organisms after injection into experimental animals or natural infection. Studies of M protein, A and A variant cell wall carbohydrates, mucopeptide, cell membrane and cytoplasmic antigens have been made (2-6). However, there is only limited information on the degradation in vivo of whole group A streptococci after intravenous injection (7) and especially of the role of the reticuloendothelial system (RES) in this process.

It was previously reported that immunologically active group A carbohydrate was detected during degradation of streptococcal cells in liver granulomata after intravenous injection of heat killed organisms into mice (8). In the present investigation, a more detailed study of the fate of streptococci trapped in the liver is presented. The role of the hepatic RES in the granulomatous tissue response was studied by light, immunofluorescent and electron microscopy. Preliminary findings are presented which characterize the streptococcal component responsible for the induction of hepatic granulomata. Details of the hepatic granulomatous response to streptococcal and other bacterial structures are reported in another paper (9).

MATERIAL AND METHODS

Preparation of Group A Streptococci and Streptococcal Components

Group A streptococci were cultured, using strain B 196, type 17, (10), heat killed and washed as previously reported (8). The preparation of cell walls, trypsinized cell walls, cell membranes, mucopeptide, C-carbohydrate and cytoplasm from group A streptococci has been described in reference 9. Some of the particulate streptococcal components were solubilized by ultrasonic treatment according to directions given in the same paper (9).

Injection of Streptococci and Streptococcal Components into Mice

Inbred albino mice (AJAX, Jackson Lab., Bar Harbour, Maine), of both sexes, weighing 25-30 gm, were used in all experiments. Forty animals received one injection of 2 mgs (dry weight) of heat-killed group A streptococci suspended in 0.3 ml physiological saline into the dorsal tail vein. The mice were exsanguinated by heart puncture at intervals from 1 hr up to 60 days after the injection, 4 animals at a given time.

Also, groups of 10 mice were intravenously injected in the same manner with 2 mgs of the different group A streptococcal fractions mentioned before. All these animals were killed 16 days after the injection.

Examination of Liver Tissue

At autopsy, one part of the liver from injected mice was fixed in formalin, embedded in paraffin and 5 μm sections cut and studied by conventional histologic procedures as earlier described (8). Another part was deep frozen in liquid nitrogen, and 3-4 μm cryostat sections were examined immunohistologically using monospecific fluorescein isothiocyanate (FITC)-labeled rabbit antibodies against streptococcal A and A-variant carbohydrates (8,9). The third part of the liver was fixed in 5 per cent buffered glutaraldehyde (pH 7.2) for 60 minutes. It was then secondarily fixed in buffered osmium tetroxide (pH 7.2, 90 minutes) and embedded in Araldit; 40-60 nm sections were cut with an ultramicrotom (LKB), stained with uranyl nitrate-lead citrate and then examined in the electron microscope (Philips, EM 200).

The liver tissues from mice injected with various components of group A streptococci were investigated by conventional histologic methods only (8,9).

RESULTS

In mice sacrificed 24 hours after the injection of whole heat-killed group A streptococci, intense granular fluorescent deposits were seen only in cells lining the sinusoids in liver sections stained with FITC labeled anti-A antibody. By electron microscopy, bacteria still possessing the characteristic morphology of cocci were found engulfed in hepatic reticuloendothelial cells (Fig. 1). As described in a previous study (8),

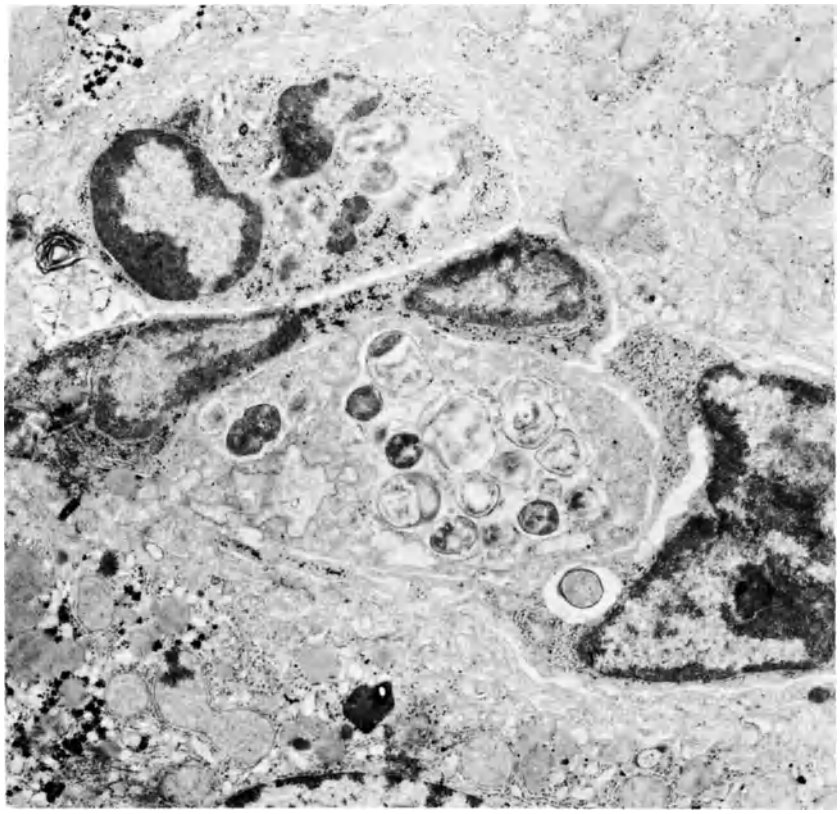

Fig. 1 Liver tissue of a mouse 24 hours after injection of heat-killed group A streptococci. Electronmicrograph, 9200 X.

after 8-10 days, cellular foci consisting predominantly of large macrophages surrounded by lymphocytes (Fig. 2a) developed; these increased in number and size up to days 16 through 20. These granulomata, rarely containing few plasma cells were shown to have an intrasinusoidal localization by electron microscopy. Early, large amounts of group A specific carbohydrates could be detected immunohistologically with FITC-labeled A antibody within these

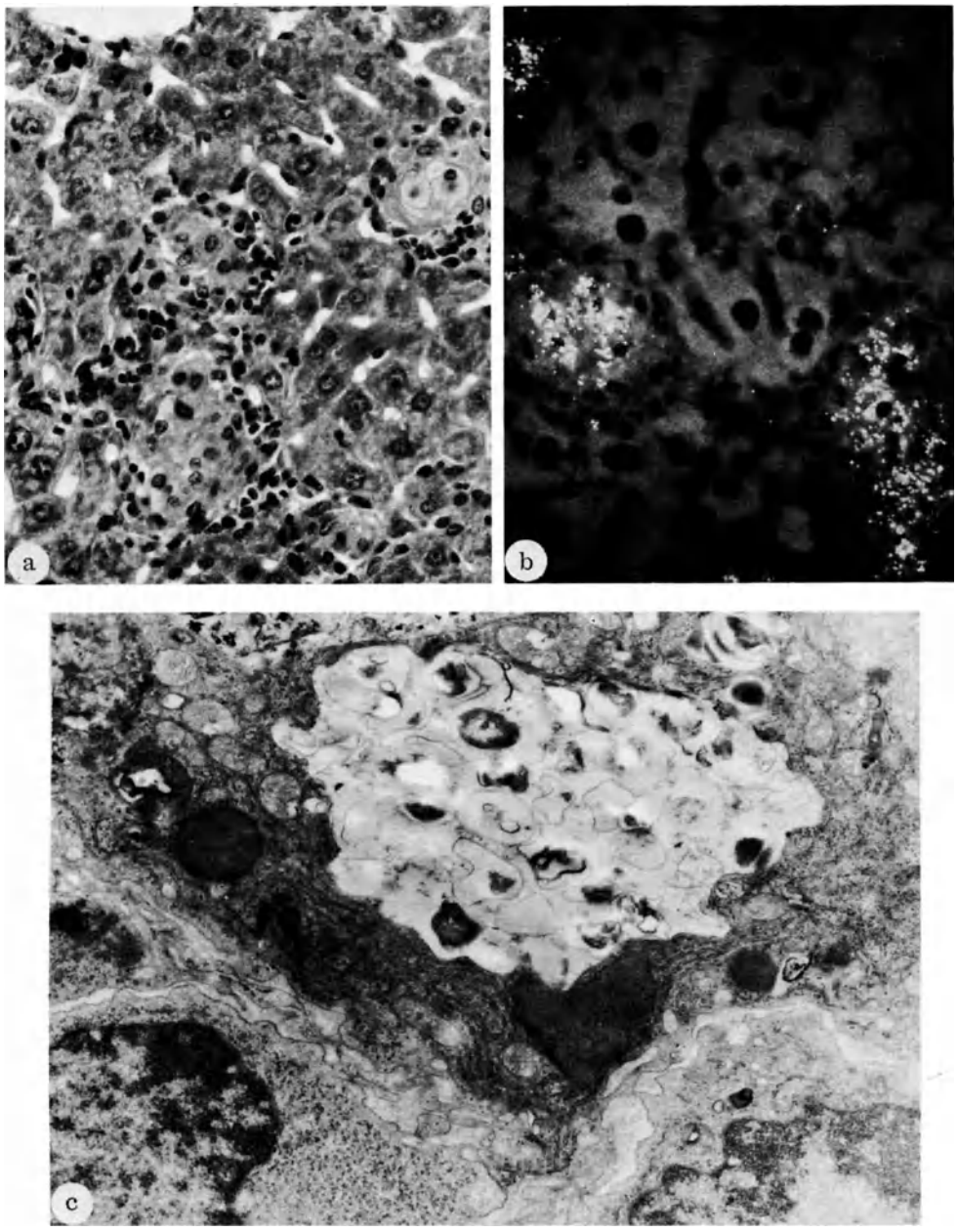

Fig. 2 a. Liver tissue from a mouse 30 days after injection of heat-killed group A streptococci. H and E, 340 X.
b. Liver tissue from the same mouse. Immunofluorescence reaction, stained with FITC labeled anti-A-variant antibodies, 320 X.
c. Liver tissue from the same mouse. Electron micrograph, 12800 X.

foci. Subsequently there was a continuous loss of group A reactive antigen during intraphagocytic degradation of streptococci; concomitantly, a steady increase in A-variant reactive carbohydrate was observed in the macrophages (Fig. 2b). Gomori staining revealed a tiny network of newly formed reticulin fibers around these cells. By electron microscopy the macrophages were found to contain phagocytic vacuoles filled with structures which appeared to be predominantly streptococcal cell walls (Fig. 2c). These cell wall remnants seemed to correspond to the granular fluorescing structures seen in immunofluorescence assays.

Experiments performed to identify the streptococcal component mainly responsible for the granulomatous liver lesions indicated that the deproteinized cell wall was almost as active as the whole streptococcus (Fig. 3). Solubilization of the cell walls by

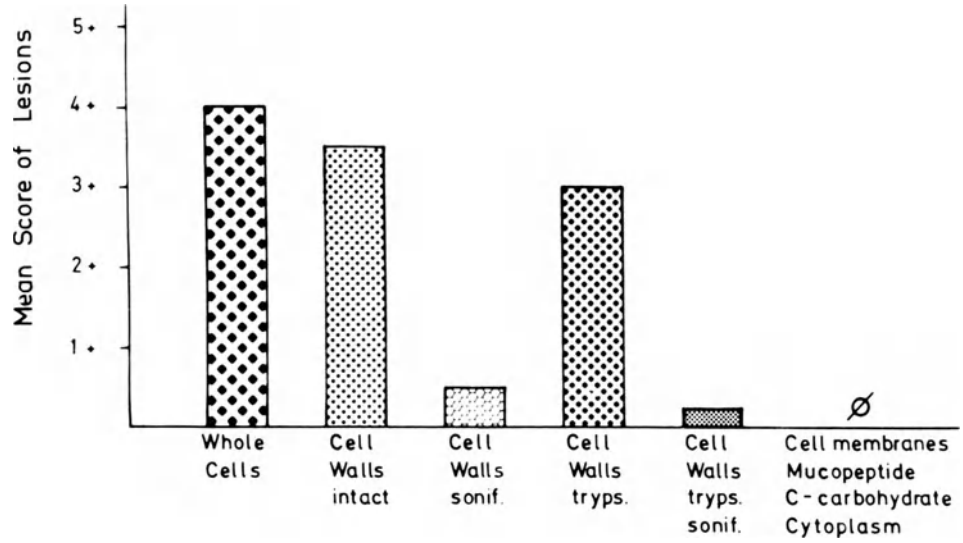

Fig. 3 Relative occurrence of liver granulomata in mice after injection of different components of group A streptococci.

ultrasonic treatment considerably reduced the induction of granulomata, indicating that particle size played a significant role in this process. Secondly, the immunochemistry of the injected particulate component represented another important factor since the wall carbohydrate-mucopeptide complex induced granulomata, whereas particulate mucopeptides and cell membranes were as ineffective as the soluble C-carbohydrate and cytoplasmic preparations in stimulating this reaction.

DISCUSSION

The results described show that heat-killed group A streptococci localized in the liver after intravenous injection into mice were phagocytized and degraded by reticuloendothelial macrophages. These macrophages according to reticulin fiber stains and electron microscopy were derived from sinusoidal endothelial and Kupffer cells (11). With lymphocytes and a few plasma cells, they formed granulomatous cellular foci which persisted for several weeks and thereafter slowly subsided during 60 days of observation. Granulocytes, known to be considerably more active than Kupffer cells in killing living bacteria (12,14), did not participate in these reactions elicited by heat-killed streptococci.

Fluorescent immunohistologic analysis of liver tissues showed that the intracellular degradation of group A streptococci was accompanied by a gradual loss of the group A specific reactivity of the cell wall C-carbohydrate with the concurrent appearance of another antigenic determinant with A-variant specificity. Similar observations have been made by other investigators who used isotope-labeled antibodies against A and A-variant carbohydrate for the identification of the corresponding antigens in tissues of mice injected with streptococcal cell walls (6,15). The modification of the A to the A-variant polysaccharide may be ascribed to the action of a particular beta-N-acetylglucosaminidase known to be present in several types of mammalian tissues and cells (13). This enzyme splits off the terminal acetylglucosamine determinant characterizing the group A carbohydrate thus liberating an oligorhamnose unit which is the determinant of the group A—variant carbohydrate.

By electron microscopy the degradation of streptococci within phagocytes could be followed to the stage of cell walls in which form streptococcal material persisted intracellularly for at least 60 days. Thus, there appear to be differences in the ability of granulocytes and Kupffer cells to digest group A streptococci since granulocytes have been shown to degrade these bacteria to a considerable extent *in vitro* within a few hours (14).

The particulate, deproteinized, streptococcal cell wall was found to be the most active component in the initiation of granulomatous cellular foci in liver. This nodular pattern of intrahepatic RES stimulation is not specific for group A streptococci since other bacteria (9) are able to elicit similar reactions. Nevertheless the toughness of the group A streptococcal cell wall, its resistance to the action of lysozyme and the relative paucity of streptolytic enzymes in mammalian tissues probably account for the long persistence of streptococcal cell wall antigens in these tissues and cells.

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LYSOSOMAL ACTIVITY IN ACTIVATED MACROPHAGES OF RAT LIVER

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This report deals with the events following phagocytosis of foreign material in Kupffer cells of rat livers. It is a study of early stages of phagocytosis of ferritin and Latex particles. All observations were made between 1 and 10 minutes after intravenous injection.

Since these phenomena have been studied by many authors and the whole topic has been excellently reviewed (Cohn and Fedorko, 1969), only a brief account of the known facts will be given; less-known phenomena, however, will be stressed.

Kupffer cells are able to engulf almost any kind of particles and types of foreign soluble proteins and mucopolysaccharides as well. Particles can be living or dead, they can vary in their surface charge, their size, wettability, etc., and most investigators have been amazed by the great efficiency with which this system works. Engulfment begins seconds after administration and within minutes, one can study almost any kind of secondary event following phagocytosis (Fig. 2). Material taken up by a macrophage is found first within a membrane-limited vacuole, called the phagosome. When the phagosome is recognized by various organelles of the macrophage, it is said to enter the digestive pathway of the macrophage. Under normal

conditions, all the phagosomes have to enter this pathway, independent of the material they contain. Essentially three organellar complexes are necessary for digestion: rough endoplasmic reticulum, Golgi apparatus and lysosomes.

Acid phosphatase is widely used at the present time as a marker enzyme for lysosomal enzymes. The generally accepted schema for the digestive pathway seems to be as follows. Acid hydrolases, formed by ribosomes, are transported via the rough endoplasmic reticulum to the Golgi complex, where they are packaged within primary lysosomes. The fusion of these latter organelles with uptake vacuoles (phagosomes) result in the formation of digestive bodies (secondary lysosomes, or phagolysosomes).

Indeed, in those Kupffer cells that actively take up foreign material in considerable quantities, we regularly observe many rough- and smooth-surfaced profiles of the endoplasmic reticulum, a richly developed Golgi complex with centrioles, microtubules, stacks of flattened cisternae and numerous smooth and coated microvesicles. There are also numerous dense granules,

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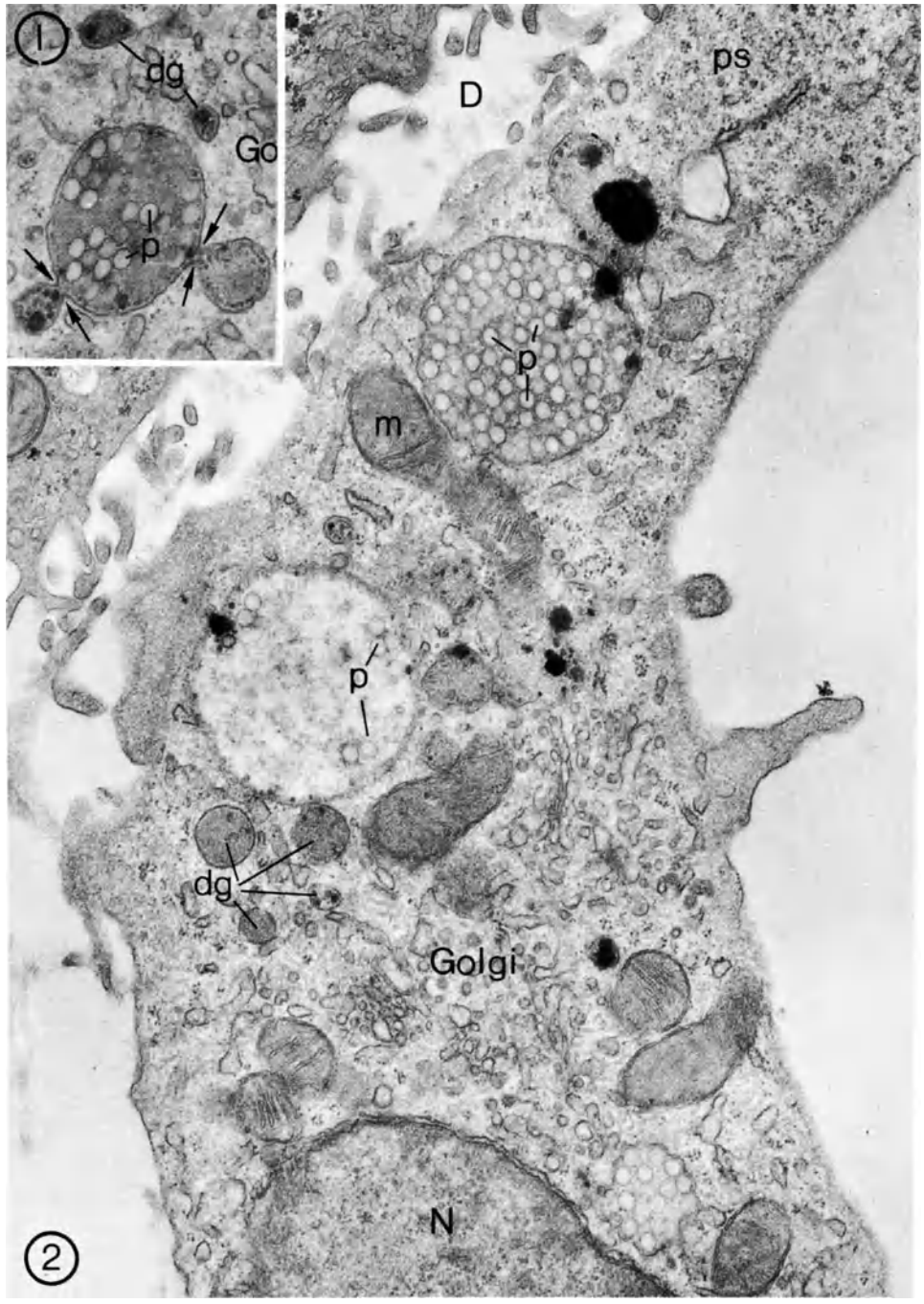
Fig. 1

A membrane-limited body, containing Latex particles (p Ø 0.1 µ) fuses with two dense granules (arrows); in its neighbourhood, dense granules (d g) and parts of the Golgi complex (Go) are found. x 28,000.

Fig. 2

Kupffer cell, 10 minutes after injection of 0.1 µ Latex particles (p). Besides the particle containing vacuoles, a well developed Golgi complex is found, as well as smooth and rough endoplasmic reticulum, dense granules (d g), mitochondria (m) and polysomes (ps). N nucleus. The space of Disse (D) separates the Kupffer cell from the hepatocyte. x 29,700.


1
dg
Go
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D
ps
p
m
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dg
Golgi
N
2

digestive vacuoles, autophagic vacuoles, multivesicular bodies and quite often we see areas containing a great number of polyribosomes (Fig. 2, 4, 6, 7).

The main task of the investigator is to reconstruct from these static pictures the dynamic series of events that lead to the digestion of the foreign material. Two points should be made at the outset. First, at our level of investigation, the events following phagocytosis seem to be identical notwithstanding the quality of the matter engulfed. Digestible proteins or undigestible Latex particles enter the same pathway.

A second and more important point is the occurrence of "mixed" vacuoles. The mixture consists of foreign and native cellular material, and heterophagocytosis thus often seems to be accompanied by autophagocytosis (Fig. 4).

Fig. 3

Vacuoles containing particles (p Ø 0.3 μ) exhibit acid phosphatase activity shortly after engulfment. x 38,500.

Fig. 4

10 minutes after injection this Kupffer cell exhibits a richly developed Golgi complex around a centriole (Ct), with numerous microvesicles and dense granules (d g). The vacuole on the top right is "mixed" (m v), it contains particles (p) as well as autophagocytic material. d b dense body, m mitochondria, D Disse's space, H hepatocyte. x 21,600.

Fig. 5

A particle (p) containing body fusing again (compare with Fig. 1) with two dense granules (d g, arrows). x 38,500.

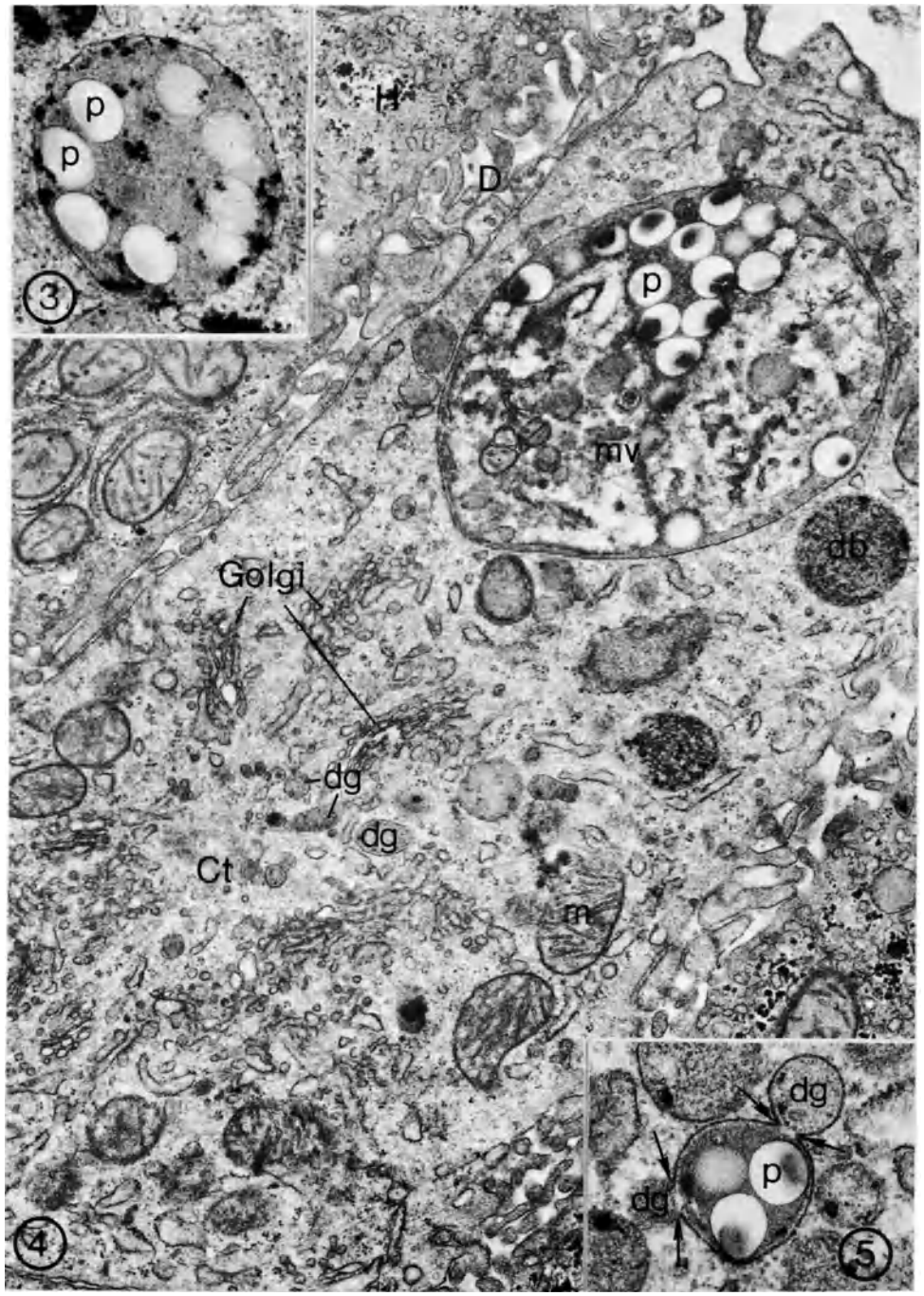

p
p
3
H
D
p
mv
db
Golgi
dg
dg
Ct
m
4
dg
p
dg
5

Fig. 6

Microtubules (mt) radiate from the centriole (Ct) to the different parts of the Golgi system: cisternae, microvesicles (mv), associated endoplasmic reticulum (er), and dense granules (dg). The arrow points to the budding-off of a microvesicle from the endoplasmic reticulum. x 25,650.

Fig. 7

Microvesicles near by Golgi cisternae may exhibit reaction product (rp) of acid phosphatase. cv coated vesicle. x 34,700.

Fig. 8

A multivesicular body, containing several microvesicles shows an invagination, suggesting "cup-like" bodies. At a, a smooth extension of the rough endoplasmic reticulum is seen, at b there is suggestive evidence of a budding-off of smooth parts of the endoplasmic reticulum, d g dense granule. x 39,600

Fig. 9

Reaction product (rp) of acid phosphatase on a multivesicular body. mv microvesicles. x 32,500.

Fig. 10

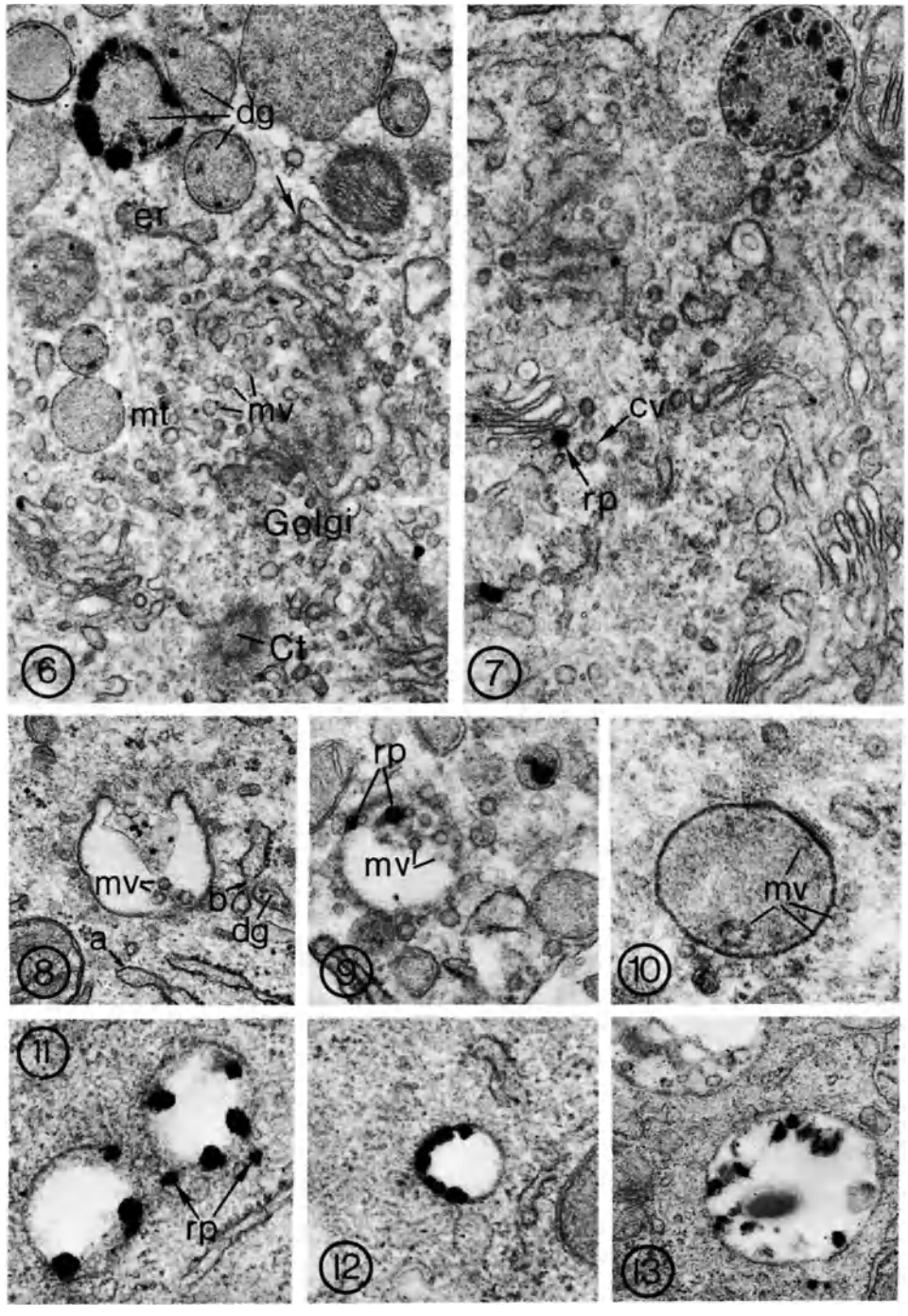
Multivesicular bodies may exhibit a plaque-like densification at their outside. mv microvesicles. x 49,000.

Fig. 11

Labelled small vesicles (rp) may occur at the outside as well as at the inside of vacuoles. x 50,000.

Fig. 12 and 13

Two vacuoles, containing labelled vesicles.
Fig. 12: x 43,700 - Fig. 13: x 26,600.


dg
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13

This phenomenon was discussed several years ago (de Duve, 1963), and a possible role of autophagocytosis in cellular regulatory mechanisms has been suggested. This may be relevant to our system, since the morphologic equivalents of active protein synthesis are indeed observed. Clusters of polyribosomes as well as extended profiles of ergastoplasm are not infrequent in actively phagocytizing cells. The most obvious reason for this would lie in the need of the cell for membrane synthesis to build up the uptake vacuoles, but until now this membrane synthesis has not been located.

Uptake vacuoles often exhibit acid hydrolase, notably acid phosphatase activity shortly after the engulfment has taken place (Fig. 3).

It has generally been claimed that, to start digestive events, fusion has to take place between primary lysosomes and phagosomes. This has been proven in neutrophils (Zucker-Franklin and Hirsch, 1964) and in eosinophils (Cotran and Litt, 1969). In mononuclear macrophages no convincing evidence for this has been obtained yet. Fig. 1 and 5 show fusion between what is currently termed a dense granule or dense body. In Fig. 6 such a dense body exhibits the reaction product for acid phosphatase. The question arises whether these fusions are the equivalent of the fusion of two digestive bodies, that is of two secondary lysosomes, or whether they represent the fusion between a primary lysosome and a phagosome. The dense granules, in this latter case, would represent primary lysosomes.

To resolve this question, a morphologist's approach is to try to trace back the origin of the dense granules by comparing cells at different stages of maturation. A dense granule, in its most differentiating form, possesses a single unit membrane enclosing a dense, amorphous matrix, separated from each other by an electron-transparent halo (Fig. 1, 2, 4, 5, 6, 7). There are often cristalloid inclusions within the matrix. The size of the dense granules averages 0.2 to 0.5 μ. In its less mature form, the granule is round to oval shaped, without a halo, and mostly without inclusions. Such bodies are located

in the immediate neighbourhood of either the Golgi apparatus or of the endoplasmic reticulum. In both locations, parts of the smooth endoplasmic reticulum sometimes in continuity with the RER, often lie very close to such "precursors", usually with an amorphous electron-dense matrix (Fig. 8). It is therefore tempting to suggest that many of the dense granules represent primary lysosomes derived directly from the smooth endoplasmic reticulum or smooth extensions of the rough endoplasmic reticulum. Such a sequence would be consistent with the model proposed by Novikoff and coworkers (1964). But what about the other localizations of acid phosphatase ? There is definitely activity in the Golgi apparatus (Fig.7), associated with the flattened cisternae and the vesicles, and there is also activity inside multivesicular bodies (Fig. 9, 12, 13). Multivesicular bodies are quite frequent in Kupffer cells. In their fully developed form they possess a number of common characteristics: a vacuole in one place shows a spiny surface coating (Fig. 10) and contains, and is surrounded by microvesicles. Multivesicular bodies have been shown to take part in the uptake of foreign proteins and particles (for example: Rosenbluth and Wissig, 1964; Fahimi, 1970). Acid phosphatase activity seems to be associated with the microvesicles, but it is an unsettled question whether Golgi-derived vesicles, laden with acid phosphatase (Fig. 7, 9, 11) fuse with or penetrate a multivesicular body; it is possible that both events take place. In any case, within 60 seconds after injection foreign substances are found inside multivesicular bodies, but never inside the contained microvesicles. Whatever the role of the multivesicular body may be, it seems to be associated with digestive processes comparable to the processes the dense granules are involved with. Finally, the Golgi complex (for the multivesicular bodies) and the smooth endoplasmic reticulum (for the dense granules) would both represent sites for the formation of primary lysosomes. A thorough discussion of the dual localization of acid hydrolases in both the endoplasmic reticulum and in lysosomes is found in the article of Fishman et al. (1969). Eventually the multivesicular bodies and the phagosomes coalesce to larger digestive bodies with their char-

acteristic content; besides the large masses of engulfed material there is a dense matrix with crystalloid inclusions, myelin figures (probably partly derived from autophagocytosis), and microvesicles. With the formation of these large digestive bodies the digestive pathway is completed. A discussion of further events, involving the effects of engulfment and digestion on overall cell metabolism and physiology, lies beyond the scope of this report.

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IN VITRO FIXATION AND DEGRADATION OF RADIOACTIVE ENDOTOXIN BY THE RES OF BCG-TREATED MICE

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It is well established by blood clearance experiments that treatment by BCG stimulates the RES and increases the resistance to tumors (1,2) and various bacterial (3,4) or viral (5) infections. However, paradoxically, this same treatment renders the host more susceptible to the lethal effect of endotoxins (6,7).

The experiments reported here show that a suspension prepared with the liver of BCG-treated mice has the ability of binding a radioactive endotoxin contrarily to what had been observed when liver of normal controls was incubated with the same antigen (8). Our results also demonstrate that the *in vitro* detoxifying capacity of a spleen extract has not been abolished by previous inoculation of BCG and that normal resistance can be partially restored by removing the spleen of mice rendered susceptible to endotoxin by such a treatment.

MATERIALS AND METHODS

In most cases, 2 mg of BCG (killed by 2% phenol) were suspended in saline and injected intravenously in a volume of 0.2 ml. Endotoxin was extracted by the phenol water procedure (9) from *S.enteritidis*, Danysz strain, and will hereafter be referred to as ASED. This

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antigen was labeled by incubating 10 mg with 1 mCi of ^{51}Cr ($Cr\ O_4\ Na_2$) according to a method previously described (10), then filtered on Sephadex G 200. Mouse livers were homogeneized as previously described (8) with a few minor modifications of the original procedure. Thus centrifugations were made at 1,000 rpm during 20 minutes, and, in certain cases, 250 mg of starch were used instead of liver homogenate, as controls. Radioactive ASED was detoxified by incubating at 37°C during 3 hours 15 µg of endotoxin in 1 ml of a saline extract corresponding to 250 mg of mouse spleen (11). Double diffusion in gel followed by autoradiography was performed according to procedures already reported (10). In all cases, the radioactive antigen was deposited in the wells. The troughs contained high titered antiserum obtained by immunizing a horse against *S.typhi* O 901. Male mice either of Swiss common stock or (CBA T6/T6 x AKR)F_1 hybrids, weighing 25 g, were used in all experiments. Splenectomy and adrenalectomy were performed under ether anesthesia. The animals were challenged by intravenous route 2 days after adrenalectomy and at various intervals after splenectomy. Deaths were recorded in 48 hours.

RESULTS

In Vitro Fixation of Endotoxin by Liver Homogenate of BCG-treated Mice

It was established by preliminary experiments that when 2 mg of killed BCG are injected intravenously, the greatest susceptibility to endotoxins is reached after an interval of 2 weeks. It was equally after a period of 14 days that the distribution of 10 µg of radioactive antigen was found to be the most modified (17% in the blood and 54% in the liver *versus* 75% and 4.5% for the controls). Therefore, in all results reported here, mice were tested 14 days after stimulation by BCG.

It has been reported that endotoxin extracted from a rough strain of Salmonellae attaches itself *in vitro* to liver homogenate of normal mice, whereas an endotoxin, such as ASED, which is extracted from a virulent smooth strain, does not (8). In view of the fact that blood clearance of ASED is increased in mice stimulated by BCG, assays were performed *in vitro* with 5µg of this radioactive antigen incubated with 250 mg of (BCG) liver suspension.

The influence of temperature and of the length of

TABLE 1

In Vitro Fixation of ASED on Liver Homogenate (BCG-treated Mice)

Influence of Temperature and Length of Incubation

Incubated with :	BCG Liver								Starch Contr.
	10 min.		30 min.		60 min.		180 min.		
	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	180min
Saline	7.9	4	8.3	5.1	11.6	5.3	31.4	6.3	-
Normal Serum 0.1 ml	13.5	4.7	30.6	5.6	35	6.9	37.8	8.9	0.16

TABLE 2

Influence of Serum on the *In Vitro* Fixation of ASED on Liver Homogenate

Comparison Between Normal and BCG-treated Mice

	Saline	Normal Serum				BCG Serum		
		0.2	0.1	0.05	0.025	0.1	0.05	0.025
Starch Control	0.5	-	-	-	-	-	-	-
Normal Liver	6	3.7	3.9	5.4	-	5.3	4.6	4.1
BCG Liver	11.5	-	21.8	17.3	10.3	24.4	18.8	9.2

time of incubation on the uptake of radioactivity was evaluated in a first experiment. As can be seen in Table 1, even after 180 minutes and in the presence of serum, only 0.16% of antigen is fixed on the control (starch pellet) and there is no significant fixation on the liver preparation at 4°C; at 37°C however, after 180 minutes, radioactive endotoxin is attached to the liver even in the absence of serum (31.4% *versus* 37.8% when serum is added). Therefore, 30 minutes and 37°C were chosen for the following assays.

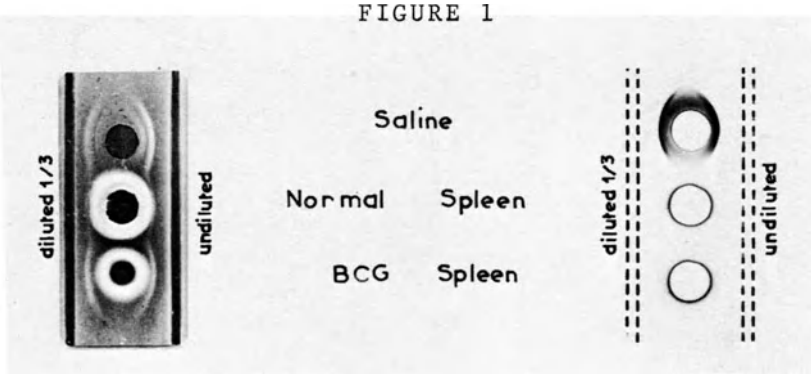
Different dilutions of serum, taken from normal mice or BCG-treated mice, were added to the antigen and to homogenate of liver of normal or BCG-treated mice. As can be seen in Table 2, a greater amount of radioactivity is attached to the liver of BCG-treated mice when 0.1 or 0.05 ml of normal serum is added. Serum from mice stimulated by BCG does not seem to induce a stronger reaction. In opposition, these sera stored at -10°C or even +4°C retained their opsonizing activity for at least 1 month. The uptake of endotoxin by liver homogenate of normal mice is significantly smaller and does not seem to be influenced by either serum. Indeed, the percentage is even higher in saline than when serum is added, perhaps because the LPS molecules are depolymerized by the latter.

Detoxification *In Vitro* by Spleen of BCG-treated Mice

It has been established that endotoxin can be degraded *in vitro* by plasma (12) and by spleen extract (13) and that, if ^{51}Cr-labeled endotoxin is used, radioactivity is removed during biological degradation (14,15,16). In the following experiments, detoxification was monitored by gel diffusion and by toxicity measurements.

Gel diffusion. A typical result is represented in Figure 1 : each well contains 2.5 µg of radioactive antigen incubated in saline, normal or BCG-treated mouse spleen. As can be seen with the normal control, there is a strong line of precipitation which corresponds to a fast diffusing unlabeled antigen and a weaker line which is closer to the well but is strongly radiopositive and corresponds to the toxic antigen. When the same antigen has been incubated in a spleen extract prepared with the organs of normal or BCG-treated mice, only the fast-diffusing unlabeled antigen can be observed. Although the wells containing the spleen extracts were surronded by a halo of protein, there existed no labeled line of preci-

FIGURE 1



Immunodiffusion patterns followed by autoradiography of radioactive endotoxin after biological degradation. Comparison between spleen of normal and BCG-treated mouse.

TABLE 3

In Vitro Detoxification of Endotoxin by Extract Prepared with Spleen of Normal or BCG-treated Mice

Toxicity was measured by injecting the antigen intravenously in adrenalectomized (Adx.) or BCG-treated mice.

Mice sensitized by :	Endotoxin incubated in:	LD_{50} (µg)	Detoxification
BCG	Saline	0.28	
	BCG spleen	>1*	× >4
Adx.	Saline	0.011	
	Normal spleen	0.71	× 64
	BCG spleen	0.59	× 53

* Only 4 dead out of 24 mice at 1 µg; the equivalent of 3 µg could not be injected because the undiluted spleen extract without endotoxin is toxic when injected intravenously.

pitation. Indeed, most of the radioactivity had been washed out (see autoradiography). These patterns are evidence of *in vitro* detoxification. Furthermore, incubation with both extracts at various dilutions showed no difference in the degree of degradation whether the spleen was recovered from normal or from mice treated by BCG. However, to evaluate more accurately this reaction, endotoxin samples treated in the same conditions were diluted and injected into mice which had been rendered susceptible by adrenalectomy or treatment by BCG.

Toxicity experiments. Table 3 represents the averages of cumulative experiments in which 388 mice were used. They were challenged with 3-fold dilutions of endotoxin varying between 0.01 and 3 µg if endotoxin was suspended in saline, and from 0.03 and 1 µg if endotoxin was suspended in spleen extract. Six mice at least were used at each dosage level.

When BCG-treated mice were challenged, the LD_{50} (saline controls) was 0.28 µg whereas it was greater than 1 µg if the antigen had been incubated with extract prepared with BCG-treated spleen. The equivalent of 3 µg could not be injected intravenously because the undiluted extract was by itself toxic. Thus, the margin of detoxification measurable by the test was too narrow. However, in adrenalectomized mice, whereas the LD_{50} (saline controls) was 0.011 µg, it was raised to 0.7 and 0.59 µg if ASED had been incubated with extract prepared with

TABLE 4

Compared Organ Weights 14 Days after Injection of BCG by i.v. or by i.p. Route

		Spleen (mg)	Liver (mg)	Lung (mg)
Controls		124	1727	190
BCG i.v.	2 mg	332	2457	208
	6 mg	492	2665	303
BCG i.p.	2 mg	152	1741	172
	6 mg	160	1858	181

spleen of normal or BCG-treated mice. Therefore, whereas the BCG-treated mouse could be killed with 0.28 µg of endotoxin, its spleen was theoretically capable of detoxifying *in vitro* between 15 and 25 µg.

Influence of Splenectomy on Hyperreactivity to Endotoxin of BCG-treated Mice

With killed BCG, organ hypertrophy was only observed if the microorganisms were injected by the intravenous route (Table 4). Thus, 40 days after the intravenous injection of 2 mg of BCG, there was a 40% increase of the liver and a 170% increase of the spleen whereas the weight of these organs was not changed significantly if the Mycobacteria had been injected intraperitoneally, even at the dose of 6 mg. Similarily, mice were much less susceptible to endotoxin if 2 mg of BCG had been inoculated by intraperitoneal route : the LD_{50} was 15 µg as against 0.28 µg when they had been sensitized by the intravenous route.

In view of these results and because it has been assumed that hyperreactivity to endotoxins was related to the granuloma formation (17), splenectomy was performed so as to remove a large amount of this tissue. Mice were operated 1, 2 or 5 days before being challenged with endotoxin; the challenge was made as usual 14 days after treatment by BCG. As can be seen in Table 5, where-

TABLE 5

Influence of Splenectomy on Hyperreactivity of Endotoxin of BCG-treated Mice

	Dose of endotoxin by iv route (µg)				
	0.3	1	3	10	30
Controls	4/8*	8/8	8/8	8/8	-
Splx. d-5	-	-	6/8	6/8	8/8
Splx. d-2	-	-	3/8	5/8	8/8
Splx. d-1	-	-	0/8	3/8	8/8

* Dead / Total

as 4 out of 8 of the unoperated controls were killed with 0.3 µg, 10 µg were needed to kill 3 out 8 mice splenectomized the day before the challenge. Thus, splenectomy seems to have restored partial resistance to endotoxin.

DISCUSSION

Susceptibility to endotoxin is increased after blockade of the RES by thorotrast (18), lead acetate (19), actinomycin D (20) or repeated injections of cortisone (21). The resistance of the host is also affected dramatically by a procedure such as adrenalectomy (22) which does not influence granulopexia. Paradoxically however, increased resistance to LPS can be observed with palmitate which inhibits the RES (23), whereas hyperreactivity to endotoxin can also be produced by bacterial agents which stimulate phagocytosis, such as *H.pertussis* (24), *Corynebacterium parvum* (25) or mycobacteria (3,4). The same pattern of response (simultaneous increase of blood clearance and of destruction of gram-negative bacteria with decrease of resistance to endotoxins) can equally be observed during the Graft Versus Host reaction (17).

These results reported here show that, similarily to what was observed with microorganisms, the blood clearance of labeled endotoxin was increased in mice inoculated with BCG and the levels of radioactivity found in their liver were higher than in those of the controls. Such differences could be produced *in vitro* : they were temperature dependent and were increased by the addition of serum. The cellular component, however, seemed to be more important than the seric factor. These experiments are being pursued using, instead of tissue suspensions, the more precise tissue-slice technique (26).

By what mechanism is endotoxin toxicity increased by injections of BCG? According to Suter, hyperreactivity to endotoxin is unrelated to delayed hypersensitivity. For instance, "cord factor", which is responsible for sensitization to endotoxin, does not sensitize to tuberculin (27). Moreover, this greater susceptibility does not seem to be the consequence of hypocorticism (see 28). It could be assumed that large doses of Mycobacteria inhibit detoxifying capacity of the RES. In our experimental conditions such is not the case. Indeed, on a weight basis, after treatment by BCG, the spleen can degrade more endotoxin than normally and, in any event, ten times the amount capable of killing the donors. It

has been suggested that, after treatment by BCG and similar situations, there appears a new population of macrophages, in the spleen and the liver, which are extremely sensitive to lipopolysaccharide (17). Our results show that splenectomy restored, to a large, extent normal resistance to endotoxin. Experiments are being continued in view of finding whether this is the consequence of the removal of a large mass of sensitized target cells or if this response is related to some other mechanism.

SUMMARY

1°) Radioactive ASED endotoxin attached itself *in vitro* to a suspension of liver of mice stimulated by BCG whereas there was no fixation if the organ was taken from a normal control. This uptake was increased if serum collected from normal or BCG-treated animals was added.

2°) Gel diffusion and toxicity experiments demonstrated that an extract of spleen of BCG-treated mice could detoxify endotoxin *in vitro* although the donors themselves had become hyperreactive to this antigen.

3°) Splenectomy performed 1 or 2 days before challenge restored partially the resistance to endotoxin of BCG-treated mice.

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CYTOCHEMISTRY OF LYSOSOMES OF THE LIVER RES DURING MALARIA INFECTION AT THE ELECTRONMICROSCOPIC LEVEL

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Malarial infection (*Plasmodium berghei*) in mice can be regarded as a model for studying drastic RES changes. We used the liver as one of the organs which undergoes very severe changes, due both to the parasitic agent and to its toxin. These structural alterations are the result of the effect of the toxicity combined with the administered infection dose (1).
In the past numerous studies dealing with the reactivity of the liver in this disease have been published (2, 3, 4, 5,).
Most of these papers revealed the activity of the RES system, and noted the accumulation of the produced malarial pigment (hemozoin) due to the breakdown of phagocytized parasitized erythrocytes.
In our system we tried to estimate the degree of phagocytosis of pigment, the fate of the ingested particles by Kupffer' cells in the liver of the mouse, and the contributions of the lysosomal enzymes (as far as possible at the ultrastructural level) to the breakdown of the hemozoin (6).

MATERIAL AND METHODS

Swiss mice were inoculated with 10^3 to 10^6 parasites (*Plasmodium berghei*) and sacrificed 2 to 28 days later by decapitation. Liver tissue was obtained as quickly as possible and transferred in small pieces into 1,7% glutaraldehyde (4°C) in 0.067M cacodylate buffer (pH. 7.0) (7), fixed for 3 hours, rinsed over night in 1,5% sucrose (4°C) in 0.014M cacodylate buffer (pH. 7,0) for cytochemical purposes. Freshly prepared 4% buffered paraformaldehyde (8) was also used as a fixative. The fixation time was 60-90 minutes at 4°C, followed by rinsing in 0.22 M sucrose before incubation. During the rinsing period tissue was chopped at 40µ with a Sorvall Tc-2 chopper (9) and incubated in various media for enzyme reactions.

Dehydration and embedding were as follows. For normal ultrastructural studies small pieces of liver tissue were fixed in 3% glutaraldehyde in 0,1 M cacodylate buffer at pH 7.2 for 2 hours, and postfixed for 1½ hour in 2% OsO_4 according to Palade, or directly fixed in the OsO_4 without prefixation in glutaraldehyde. Following dehydration in ethanol the tissue was embedded in EPON 812. Thin sections for electron microscopy were cut with a Reichert OM U2 or LKB microtome, and examined unstained or uranyl-stained under a Philips EM 300 (40-60 KV).
For light microscopical investigation, the liver tissue was frozen and sectioned at 10µ, fixed, and lysosomal activity determined according to the methods of Gomori (10) or Barka (11) (acid phosphatase), Goldfischer (12) (arylsulphatase), Wachstein (13) (esterase). Incubation times varied between 30-60 minutes. For fine structural cytochemical studies we used slightly modified techniques of Barka (14) for the acid phosphatase, Hopsu-Havu (15) for arylsulphatase, Miller (16) or Hugon (17) for E600-resistent esterase.

Substrate free media and denatured sections were used as controls. The incubation times were 30 to 60 minutes at 37°C.

RESULTS

The light microscopical histochemical findings revealed an increase in acid phosphatase and arylsulphatase in the phagocytic cells of the sinusoidal endothelium (s.e.). In the liver cells there was no remarkable increase in lysosomal elements, or in esterase activity. With the exception of the Barka (11) method, it was difficult to state the exact localization of the activity of this enzyme and its relation to the phagocytized pigment with the lead precipitation techniques. Therefore,fine structural localization was used. In the electron micrographs of normally embedded material, the shrunken liver cells were seen to be filled with lipid droplets. The endothelium appeared to be highly irritated. The space of Disse and the interstices between the hepatocytes were widened. The sinusoids were dilatated and engorged with cells. Most of these lining cells contained phagocytized hemozoin (malarial pigment), or entire parasitized and normal erythrocytes (fig. 1).
During the course of infection the activity of acid phosphatase increased, particularly in the highly irritated s.e. cells (18). The phagocytic cells contained a large amount of pigment and ingested remains of parasitized erythrocytes. The rectangular hemozoin particles were found to be located in digestive vacuoles. An intense activity of acid phosphatase was found to be localized between the crystalloid pigment particles (fig. 2).
Another lysosomal enzyme,i.e. arylsulphatase was hardly noticeable in control animals, and conspicuously absent in the s.e. cells. During the course of the infection, however, a gradual increase in

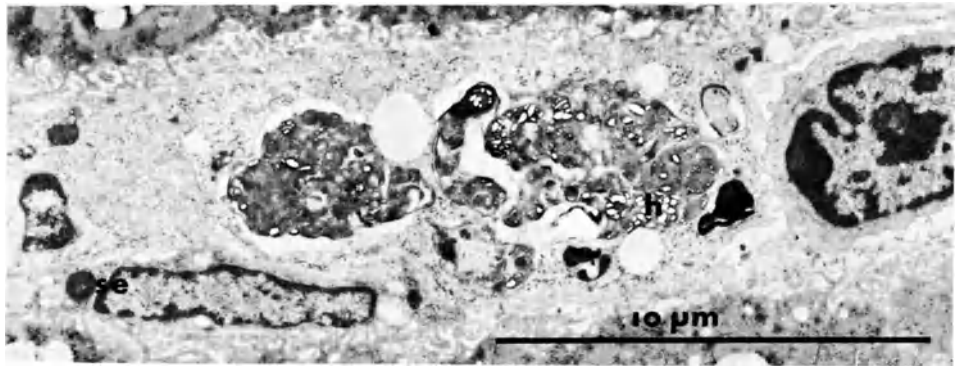

fig. 1. se = sinus endothelium h = hemozoinparticles
Note the phagocytized material with malaria pigment
(partly dissolved by staining with leadcitrate).

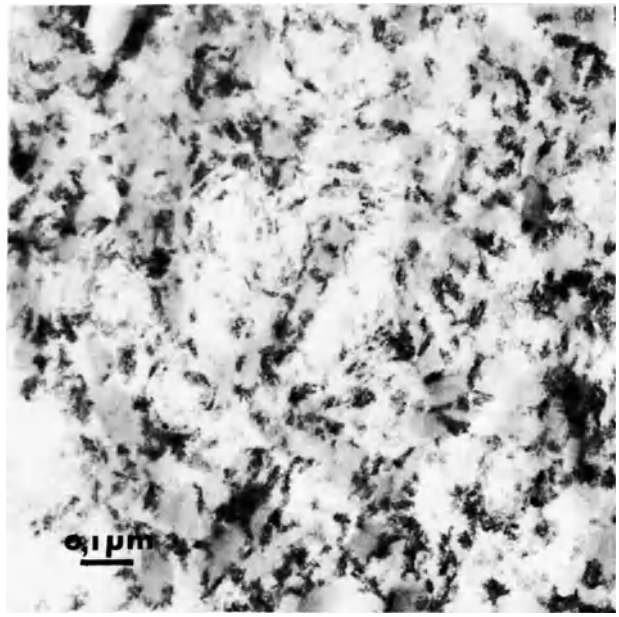

fig. 2. A high magnification shows the precipitation of
acid phosphatase between the grey crystalloid pigment
(uranyl).

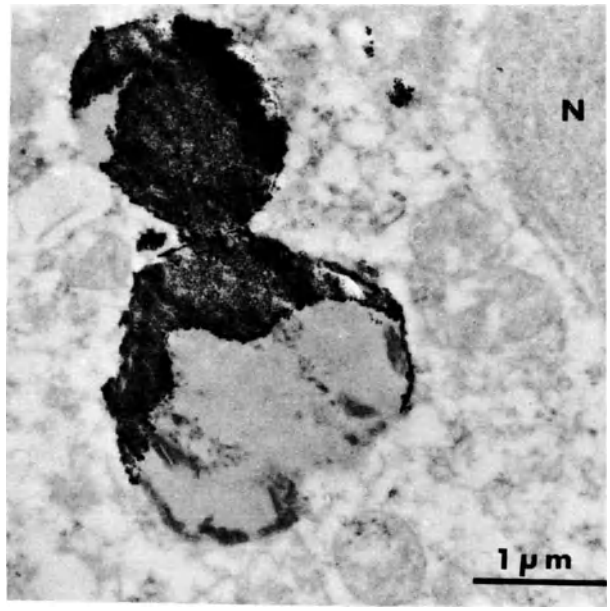

fig. 3 N = nucleus
Note the 2 big digestive vacuoles with the fine precipitates (Ba method) (uranyl)

enzyme activity occurred in the digestive vacuoles, which contained the pigment granules. Although also associated with hemozoin, the arylsulphatase activity differed with respect to two features from that of acid phosphatase.
First: whereas all digestive vacuoles showed acid phosphatase activity, not all of them showed arylsulphatase activity.
Secondly: whereas acid phosphatase activity was always regularly distributed within the digestive vacuole, the arylsulphatase activity was confined to selected areas (fig. 3).
A third enzyme, i.e. the E600-resistent esterase, differed from the other ones. We did not succeed in demonstrating a remarkable esterase activity in the phagolysosomes (fig. 4).
There was only a little increase in activity of this enzyme during the infection, and no fusion was seen between phagosomes containing hemozoin and the lysosomes with primary esterase activity. This enzyme tended to gather around the digestive vacuoles, without being involved in the breakdown of the ingested material.

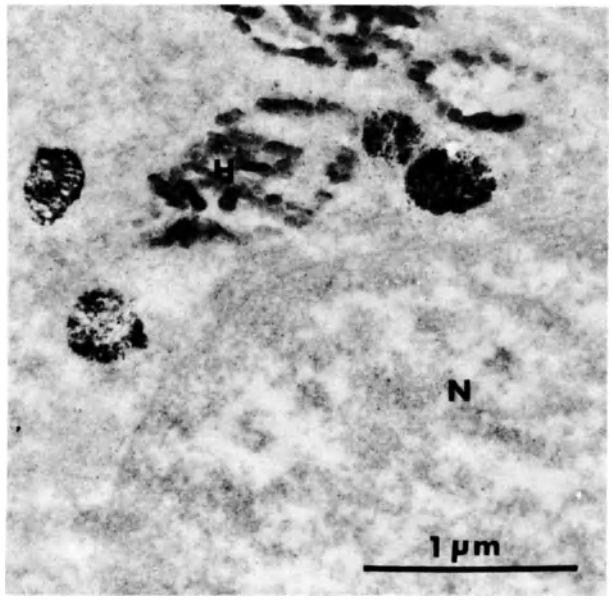

fig. 4. N = nucleus H = malaria pigment
No precipitation of esterase activity is noted within the pigment accumulations (unstained-40kV).

DISCUSSION AND CONCLUSIONS

The RES of the liver, i.e. the lining sinusoidal endothelium, plays an important role in the removal of worn out and non-self material from the circulating blood. During the course of infection with *Plasmodium berghei* not only the number of macrophages, but also their phagocytic activity, increases enormously. These cells phagocytize both parsitized erythrocytes and cell debris occurring as a result of cell degeneration due to severe malarial infection. As judged from light microscopy, phagocytized cell components appear to be degraded rather quickly; malarial pigment, however, is found to be stored in the macrophages for a long time.

Malarial pigment is a product of the malaria parasite which digests the host cell's hemoglobin and stores the useless remnant as a crystalloid pigment, the hemozoin. It has already been stated by other authors (19,20, 21) that hemozoin particles are stored within the malarial parasite *P. berghei* in scattered small vacuoles separated from the parasitic cytoplasm by a membrane.

On our electron micrographs, macrophages show ingested cell debris, intact parasitized erythrocytes, and remnants of both parasites

and their host cells. After degradation of structured cell components the phagosomes were found to be filled with an amorphous material. The hemozoin which first occurs scattered in the cytoplasm of the macrophages appears to be collected in phagosomes of varying sizes, because most of these pigment containing phagosomes are larger, and the pigment is accumulated in a much higher density than in the original hemozoin containing vesicles of the parasite. The acid phosphatase appears to play an important role in the degradation procedure. Primary lysosomes, always acid phosphatase positive were found to fuse with the phagosomes to form phagolysosomes (22). In these phago-lysosomes the activity of this enzyme was regularly distributed throughout the entire vacuole. This holds true also for phago-lysosomes which contain mainly hemozoin. In these vacuoles the electron dense precipitations can be easily distinguished from the less electron dense hemozoin crystalloids. The role of the acid phosphatase is further indicated by the remarkable increase in enzyme activity observed during the course of the malarial infection.

Whereas other cell components disappeared rather quickly, hemozoin was found to be stored for a long time in the macrophages. This may indicate that the acid phosphatase is not able to degrade hemozoin completely. Therefore, it is a possibility that the arylsulphatase is involved in hemozoin degradation. An arylsulphatase activity is hardly detectable in the normal s.e. cells, and its activity remains low during the first days of infection. During the course of the infection both an increasing number of primary lysosomes and of phago-lysosomes with arylsulphatase activity could be noted; but the activity remained lower than that of the acid phosphatase, and there were differences in the distribution. Firstly, not all lysosomes and phagolysosomes exhibited aryl sulphatase activity, and secondly, in instances of a positive reaction, the phago-lysosomes contained "white areas" of different size. In most instances the digestive vacuoles showed substrate precipitation in the areas where malarial pigment was accumulated; thus precipitated granules masked the hemozoin.

It appears that the activity of type B arylsulphatase, which particularly acts at pH of 5.5 (23) and lower, is lower than the activity of the acid phsophatase. Although arylsulphatases types A and B can be separated by paper electrophoresis, their general properties do not appear to differ significantly from each other. Possibly both types act as sulfotransferases (24). Little is known, however, concerning the physiological function of these enzymes, e.g. whether they act under certain conditions as hydrolytic enzymes, or as transferases. In the case of malarial infection it appears possible that type B of this enzyme acts only during a certain phase of the degradation procedure in order to carry out a special reaction, e.g. on a certain (sulphate?) group.

This could explain why, in contradiction to the regularly dis-

tributed activity of acid phosphatase, the arylsulphatase is irregularly distributed not only within one phago-lysosome, but also between the different phago-lysosomes, and shows different intensity of activity. It is a moot point whether the aryl sulphatase acts only for a short time after the fusion of the primary lysosome with a phagosome which is primarily inactive for arylsulphatase, or whether thereafter the activity may decrease once the special degradation step has been carried out. The question remains open, whether the arylsulphatase is a pre-existing, but inactive, enzyme activated by specific compounds of phagocytized material, or whether it is an enzyme which arises adaptively.

Concerning the E600-resistant esterase, only a gathering of enzyme positive lysosomes in the vicinity of some digestive vacuoles could be noticed. A fusion of these two structures could not be observed regularly, at best incidentally. We therefore believe that this enzyme is not involved in the degradation of ingested particles, particularly not with the breakdown of hemozoin.

It may be concluded from these results that the RES of the mouse liver can react in different ways to a given stimulus. In the case of malarial infection, and with regard to the phagocytosis, it can be stated that malarial pigment is not as quickly degraded as other structural cell compounds. It may be assumed from the distribution pattern and their activity that the three main lysosomal enzymes do not act in the same way. Since a certain preference for the phagocytized material could be noted, particularly for malarial pigment, the RES cells are shown to be able to react uniformly to the stimulus.

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SEQUENCE OF EVENTS LEADING TO THE METABOLIC STIMULATION IN PMN LEUCOCYTES DURING PHAGOCYTOSIS

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The most dramatic changes induced by phagocytosis in PMN leucocytes are: 1) increase of oxygen uptake, insensitive to cyanide, rotenone, Antimicin A and amytal; 2) increase of H_2O_2 production; 3) increase of glucose oxidation through the HMP pathway (1-8). Similar changes are induced by treatment of leucocytes with surfactants (9,10) or antileucocyte antibodies (11).

In this paper we will describe the factors which provide a link between these reactions, our attention being focused essentially on the following points:

1) Enzymatic basis of the metabolic stimulation.
2) Properties of the enzyme, whose activation initiates the stimulation process.
3) Mechanism of activation of this enzyme.

1. The enzymatic basis of the metabolic stimulation.

Two main hypotheses have been proposed to explain the metabolic stimulation during phagocytosis. The first one directly links the increase of respiration to the HMP pathway activity (3,4,10, 12-20). According to this hypothesis an activation of a granule-bound NADPH oxidase is the key event leading to a rise in oxygen uptake, H_2O_2 production and generation of $NADP^+$. The increased availability of the oxidized pyridine nucleotide, limiting factor in the HMP pathway, would stimulate the glucose oxidation.

The other hypothesis postulates an indirect linkage between the enhancement of respiration and HMP pathway. The increase of respiration and H_2O_2 production would be supported by an activation of a granule-bound NADH oxidase (8,21-24) utilizing the glycolytic NADH. In this view, the increased availability of $NADP^+$ for the HMP pathway stimulation would be provided by additional reactions catalyzed by: a) an NADPH-linked lactate dehydrogenase, utilizing pyruvate available in glycolysis (21,22), or b) an NADPH-linked glutathione reductase, coupled to a glutathione peroxidase dealing with the H_2O_2 produced during the oxidation of NADH (25).

To decide which one of the two hypotheses is more reliable, we have analyzed the main features of the oxidation of NADPH and NADH by granules of leucocytes at rest or under phagocytosis. Leucocytes from guinea pig exudates have been used throughout the research. Some experiments have been carried out with peroxidase-negative leucocytes of human blood. The following results have been obtained.

a) The comparison between the activities of NADPH-and NADH-oxidase has been carried out polarographically and spectrophotometrically by using granule fractions isolated from the same number of resting or phagocytizing cells. Table I shows the rate of oxygen uptake by granules at pH 5.5, in the presence of two different concentrations of NADPH and NADH. The oxidation of both nucleotides by granules of leucocytes phagocytizing either latex spherules or bacteria appears to be stimulated, as compared to that catalyzed by granules of leucocytes at rest.

The rate of NADPH oxidation is higher than that of NADH, at the two substrate concentrations, both in granules derived from leucocytes at rest and under phagocytosis.

A comparison between the rate of oxygen uptake by the whole phagocytes and the activity of the two oxidases (Table I) shows that both enzymes could theoretically mediate the respiratory response in phagocytizing cells.

b) Since the respiratory increment in phagocytes is virtually insensitive to KCN, we have investigated the effect of this inhibitor on the oxidation of NADPH and NADH. The oxidation of NADPH by isolated granules of PMN leucocytes at rest (measured as oxygen uptake) is inhibited 30-50% by 2 mM KCN, whereas the activity of the oxidase of granules from phagocytizing cells is practically not

TABLE I

RESPIRATION OF PMN LEUCOCYTES AND NADPH AND NADH OXIDASE ACTIVITY OF CORRESPONDING GRANULES (natoms oxygen/minute)

	Resting	Phagocytizing	
		+ Polystyrene	+ Bacteria
Whole cells° ($7.8 \cdot 10^6$)	6	18	33
Granules°° (from $7.8 \cdot 10^6$ cells)			
+ 0.4 mM NADPH	15	-	185
+ 2.5 mM NADPH	50	250	420
+ 0.4 mM NADH	5	-	45
+ 2.5 mM NADH	17	65	190

°Krebs-Ringer phosphate buffer, pH 7.4; 2 mM KCN. Phagocytosis: 5 minutes at 37°. Polystyrene spherules (0.81 μ diameter) or *B. subtilis*/leucocyte = 100:1.

°°65 mM phosphate buffer, pH 5.5; 125 mM sucrose; 0.5 mM $MnCl_2$; 2 mM KCN. Granules isolated from the postnuclear supernate at 20,000 g for 20 minutes.

inhibited (fig. 1). When the resting granules are assayed for their ability to oxidize NADH (from 1 to 4 mM) an inhibition of 60-80% is found. On the other hand, when the NADH oxidation is performed by granules of phagocytizing cells an inhibition of less than 10% is observed.

Similar results have been obtained by measuring the activity of NADH- and NADPH-oxidase in a range of pH's from 5.5 (condition of maximal activity) to 7.2. Since the measurment of NADPH and NADH oxidation as rate of oxygen uptake in absence of KCN might be influenced by the simultaneous production of oxygen from H_2O_2 catalyzed by endogenous catalase (19, 25), we have checked the KCN inhibition also spectrophotometrically (Table II). To allow the detection of absorbancy at 340 nm in cuvettes of 1 cm light-path, lower concentrations of the reduced pyridine nucleotides have been used (0.166 mM). Again a marked inhibition of the nucleotide oxidation (about 70%) is observed with granules of resting leucocytes. When using granules of phagocytizing leucocytes, the oxidation of NADPH appears to be virtually unaffected by KCN, whereas

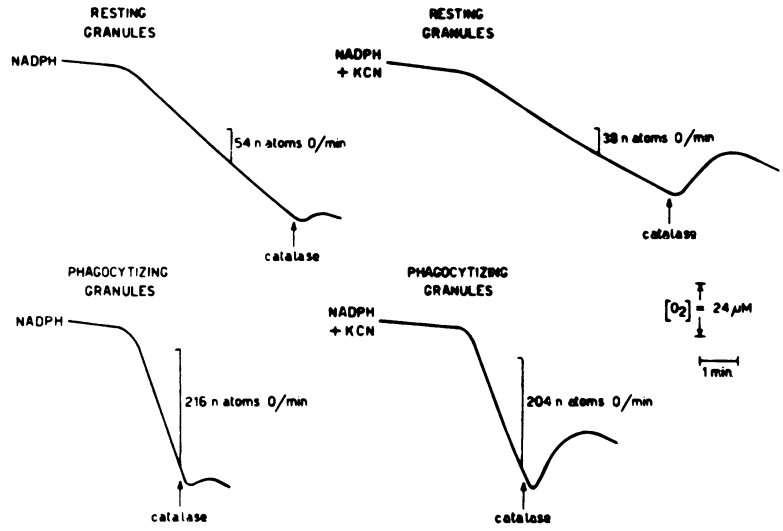

Figure 1.- Effect of KCN on NADPH oxidase activity of granules from resting and phagocytizing leucocytes.
Assay medium as reported in Table I. Resting granules: 4 mM NADPH, 100 µg protein/ml; phagocytizing granules: 1 mM NADPH, 50 µg protein/ml. Similar inhibitions were observed at any concentration of NADPH from 1 to 4 mM. Catalase is added to show accumulation of H_2O_2.

TABLE II

EFFECT OF CYANIDE ON THE OXIDATION OF NADPH AND NADH BY GRANULES OF RESTING AND PHAGOCYTIZING LEUCOCYTES

	Resting		Phagocytizing	
	- KCN	+ KCN	- KCN	+ KCN
NADPH oxidase	0.26± 0.03	0.07± 0.02	0.89± 0.19	0.81± 0.20
NADH oxidase	0.15± 0.02	0.05± 0.01	0.36± 0.03	0.24± 0.02

Spectrophotometric determination at 340 nm. Assay media as reported in Fig. 1, 0.166 mM NADPH or NADH. Enzyme activities are expressed as µmoles of pyridine nucleotide oxidized/3 minutes/mg protein (average values of at least 5 experiments ± S.E.).

that of NADH is slightly inhibited (about 30%).

In conclusion, these results show that, when the cell passes from the resting state to phagocytosis, the oxidation of NADPH and

NADH becomes insensitive to KCN. This finding might be explained by assuming that, beside the specific oxidase(s), other enzymes, i.e. KCN-sensitive haemoproteins, contribute to the oxidation of reduced nucleotides. The relative contribution of these enzymes would be rather relevant when the specific oxidases disply low activity (resting leucocytes), while their contribution would become almost undetectable when the oxidases have been activated (phagocytizing leucocytes). An alternative explanation is that the oxidation of the reduced pyridine nucleotides is performed by specific oxidases, whose insensitivity to KCN appears only after the enzymes have been modified in the process of phagocytosis.
c) Both oxidases, associated with isolated granules, are activated in phagocytosis and are cyanide insensitive. Furthermore, their activities could account for the burst of respiration. Analysis of the kinetic properties of these enzymes and a precise measurement of the concentration of NADPH and NADH in intact cells, should help establish which one of the oxidases is likely to be functional within the leucocyte.

We have measured the KCN-insensitive NADPH and NADH oxidase activities in the presence of different concentrations of substrates, from 0.2 to 4 mM. Data were statistically elaborated as suggested by Wilkinson (27). It can be seen from Table III that the kinetic properties of NADPH oxidase markedly change during phagocytosis. In fact, the enzyme of granules obtained from phagocytizing leucocytes has a K_m for NADPH, which is about ten-fold lower than

TABLE III

Km FOR PYRIDINE NUCLEOTIDES OF NADPH OXIDASE, NADH OXIDASE AND LACTATE DEHYDROGENASE OF GUINEA PIG LEUCOCYTES.

	Resting	Phagocytizing
NADPH Oxidase	$2\text{-}5 \cdot 10^{-3}$ M	$2\text{-}6 \cdot 10^{-4}$ M
NADH Oxidase	-	$2\text{-}6 \cdot 10^{-3}$ M
Lactate dehydrogenase	-	$3\text{-}6 \cdot 10^{-6}$ M

The oxidases were assayed polarographically in the presence of 2 mM KCN, as reported in Table I.
Lactate dehydrogenase activity was measured according to Beck(29).

that of resting granules. Furthermore, the V_{max} of the former was always found to be at least 4 times higher than that of the latter, on the protein basis. Table III also shows that the K_m of NADH oxidase of granules from phagocytizing leucocytes is tenfold higher than that of NADPH oxidase.

The concentrations of oxidized and reduced pyridine nucleotides in leucocytes at rest and during phagocytosis are reported in Table IV. These data clearly indicate that the oxidation of NADH in intact cells cannot be performed at a suitable rate by the NADH oxidase. In fact, the concentration of NADH in phagocytizing leucocytes is two order of magnitude lower than that required for maximal activity of the enzyme. Furthermore, Table IV shows that the concentration of NADPH in resting leucocytes is very far from that required for the maximum activity of the granule-bound NADPH oxidase. In cells at rest the rate of the enzyme reaction would be very low. As the process of phagocytosis brings about a modification of the kinetic properties of NADPH oxidase, conditions for maximal activity of the enzyme in intact cells are met by the actual concentration of NADPH, which is in the range of K_m values. That NADPH and not NADH is likely to be oxidized in vivo is also supported by the finding that in phagocytizing leucocytes the $NAD^+/NADH$ ratio does not change, whereas, the $NADP^+/NADPH$ ratio is three-fold increased (Table IV and ref.28).

TABLE IV

PYRIDINE NUCLEOTIDE CONTENT OF RESTING AND PHAGOCYTIZING LEUCOCYTES.

	Resting		Phagocytizing	
	mM	Ratio	mM	Ratio
NAD^+	0.560		0.580	
		7.1		7.6
NADH	0.079		0.076	
$NADP^+$	0.041		0.084	
		0.11		0.31
NADPH	0.350		0.273	

NADPH and NADH were extracted with 0,33M KOH in ethanol, NAD^+ and $NADPH^+$ with 0.6M perchloric acid. The pyridine nucleotides were assayed in the neutralized extracts according to Estabrook and Maitra(53) Assumed average volume of PMN leucocyte = 270 μ^3.

The role played by the two enzymes in intact leucocytes depends not only on the affinity for the substrates, but also on their actual concentration which is controlled by concomitant metabolic pathways. NADH oxidase would utilize the glycolytic NADH. Thus, competition for NADH would occur between NADH oxidase and lactate-dehydrogenase. By comparing the K_m for NADH of the two enzymes in resting and phagocytizing leucocytes we have found values of 3-6 x 10^{-6} M for lactate dehydrogenase and of 2-6 x 10^{-3} M for NADH oxidase (Table III). These figures are only slightly affected by pH variations from 5.5 to 7. In human blood leucocytes the K_m of lactate-dehydrogenase has been found 5 x 10^{-5} M (29) and that of NADH oxidase 4 x 10^{-4} M (24). On the basis of these data, one wonders how, in phagocytizing leucocytes, the NADH of cytosol would be preferentially oxidized by NADH oxidase.

d) The experimental data presented here as well as those previously reported strongly support the conclusion that the main event responsible for the stimulation of respiration is the activation of NADPH oxidase. This activation allows a simple and a direct link between this stimulation and the activation of glucose oxidation through the HMP pathway. On the contrary, by assuming that an activation of NADH oxidation is responsible for the burst of respiration, an indirect mechanism for generation of $NADP^+$ is required. The quantitative adequacy of the ancillary reactions proposed for the oxidation of NADPH is, however, liable to some criticism:

i) The activation of NADPH-linked lactate dehydrogenase by an intracellular acidification caused by increased glycolysis (21, 22) is not feasible, since the increase of the rate of glycolysis has been proved to follow the burst of respiration of leucocytes upon the addition of bacteria (17). In the case of metabolic stimulation operated by saponine, the respiratory increment is even accompanied by a depression of the rate of glycolysis (10).

ii) There is no correlation between the amount of lactate produced during phagocytosis and the generation of $NADP^+$ required for the oxidation of glucose through the HMP pathway, the lactate being in large deficit (5).

iii) An NADPH linked lactate dehydrogenase is not operating in human leucocytes as shown by the direct assay of the enzyme (24) and by following the fate of tritium labelled Carbon-1 of glucose (30).

iv) The reactions of the glutathione cycle coupled to NADH

oxidation can be summarized as follows:

(1) $NADH + H^+ + O_2 \longrightarrow NAD^+ + H_2O_2$

(2) $H_2O_2 + 2\ GSH \longrightarrow 2\ H_2O + GSSG$

(3) $GSSG + NADPH + H^+ \longrightarrow 2\ GSH + NADP^+$

(4) $NADP^+ + G\text{-}6\text{-}P \longrightarrow NADPH + H^+ + 1/2\ CO_2$

If H_2O_2 were preferentially utilized in reaction (2), the stoichiometry of the overall process would be 1 mole of CO_2 per 2 mole of O_2 consumed. Therefore, when the respiratory stimulation is very efficient and glucose is predominantly catabolized via the HMP, the respiratory quotient should be closed to 0.5. On the contrary, experiments carried out in this laboratory, both by standard manometric technique and by comparing the production of $^{14}CO_2$ from ^{14}C-U-glucose with oxygen uptake (Rossi, unpublished), have consistently yielded a respiratory quotient of 1.0 in resting and phagocytizing leucocytes. The involvement of the glutathione cycle in H_2O_2 utilization and $NADP^+$ production would be compatible with a respiratory quotient of 1, only if H_2O_2 were supplied by a concomitant and faster oxidation of NADPH catalyzed by the activated NADPH oxidase.

v) In short time experiments, when oxygen uptake is stimulated by phagocytosis, H_2O_2 is stoichiometrically accumulated in the presence of KCN (26). This finding questions the relevant utilization of H_2O_2 by the glutathione cycle, which is not inhibited by KCN (24,31).

e) The insensitivity to KCN of the NADPH oxidation, at least in phagocytosis, rules out the possibility that such reaction is catalyzed by a cyanide-sensitive peroxidase, as suggested by Roberts et al.(32). Moreover, conclusive evidence that the NADPH oxidation is not catalyzed by myeloperoxidase is provided by the metabolic response to phagocytosis of human leucocytes with ereditary myeloperoxidase deficiency. Lehrer et al.(33,34) have reported that myeloperoxidase-deficient leucocytes display normal morphological and metabolic response to phagocytosis, albeit their bactericidal activity is impaired. We have found a 24-year old man, whose neutrophiles and monocytes were lacking myeloperoxidase, as demonstrated hystochemically and biochemically. In the presence of bacteria, the leucocytes of this patient exhibit a normal stimulation of respiration, H_2O_2 production and glucose oxidation via the HMP pathway. Measurement of NADPH oxidation by

isolated granules reveales normal levels of NADPH oxidase activity and normal activation of the enzyme by phagocytosis.

2. The association of NADPH oxidase to the granules of PMN leucocytes.

After their first isolation by Cohn and Hirsh (35), a great deal of attention has been paid to the characterization of the granules of PMN leucocytes.

Morphological, hystochemical and biochemical evidence has been provided to show that (36-42):
a) distinct types of granules are present in heterophil leucocytes, b) in addition to lysosomal acid hydrolases, several components not usually found in the lysosomes of other cells (peroxidase, cationic protein), are present in these granules; c) not all the granules can be qualified as lysosomes.

We have investigated the problem of the association of the KCN-insensitive NADPH oxidase to the granules in a way similar to that followed to study the lysosomal enzymes. The mode of association of the oxidase to the granules is relevant for the mechanism of its activation during phagocytosis. In fact, while the hydrolytic enzymes are discharged from the granules in the process of phagocytosis (14,43-45), NADPH oxidase is activated without release into the cytoplasm (4,14,16).

The latency of NADPH oxidase, in comparison to that of myeloperoxidase and acid phosphatase, has been investigated by measuring the activating effect of Triton X-100, a nonionic detergent.

Figure 2 shows that the specific activity of acid phosphatase and peroxidase is strongly enhanced by Triton X-100. On the contrary, the specific activity of NADPH oxidase is only slightly increased by the action of the detergent.

The activation of acid phosphatase and peroxidase is a typical feature of lysosomal enzymes, which are segregated within a lipoprotein membrane. The lack of activating effect on NADPH oxidase may be explained by one or more of the following assumptions:
a) the enzyme is located in granules different from lysosomes;
b) the enzyme is segregated within a granule whose membrane provides no efficient barrier to NADPH entry; c) the enzyme is bound to the membrane of a granule in a way to be always available to the substrate.

The release of the enzymes from the granules has been followed after treatment with ionic and nonionic detergents, and after freezing and thawing. The results (Fig.3) show that 1) acid

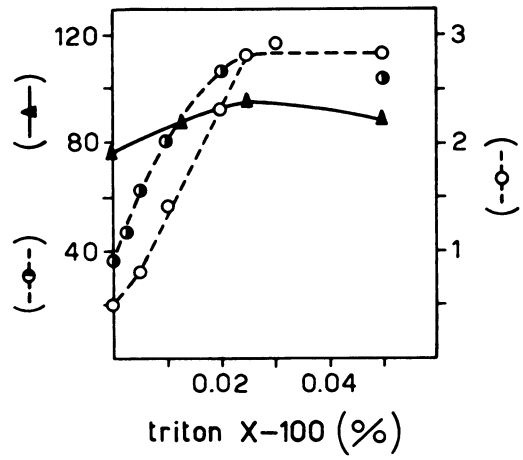

Figure 2 - Specific activity of peroxidase (-o-),acid phosphatase (--0--),NADPH oxidase (-▲-) of PMN leucocyte granules in the presence of different amounts of Triton X-100.
Peroxidase was assayed with the guiacol test (54),acid phosphatase with β-glycerophosphate as substrate (55) and NADPH oxidase as reported in Table I.

phosphatase is both activated and released by all the treatments, although to a different extent; 2) myeloperoxidase is scarcely released by any treatment except for the cationic detergent,which, in addition, produces an enormous rise in activity of the enzyme, higher than that produced by any other treatment; 3) NADPH oxidase is released only by Cetyl trimethyl ammonium, but this release is not associated with any substancial increase of enzyme activity. The other treatments are not able to solubilize the enzyme and, at the same time, do not produce any significant change of its activity.

From these results one may conclude that NADPH oxidase of resting granules is not a latent enzyme by conventional meaning. The release of the enzyme by the cationic detergent without activation suggests that the enzyme is fully available to the substrate also when it is associated to the granules. We favour the view that the enzyme is localized on the outer surface of the granule.

Phagocytosis and other treatments of leucocytes induce an in-

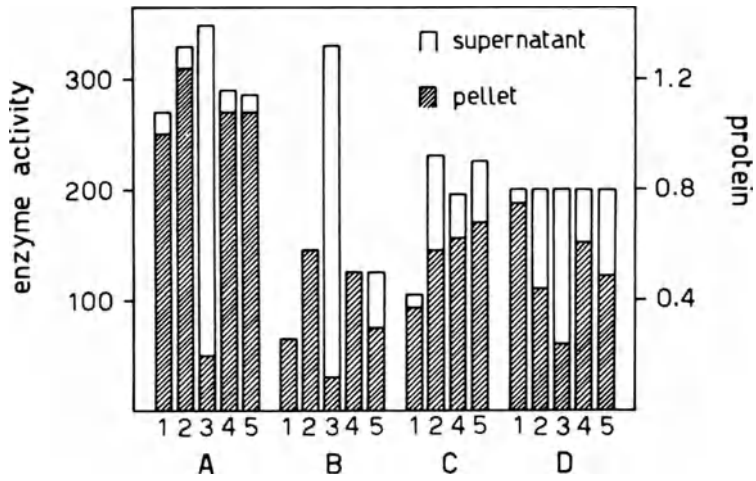

Figure 3 - Release of NADPH oxidase (A), peroxidase (B), acid phosphatase (C) and protein (D) from isolated leucocyte granules. 1 = Control; 2 = 0.05% Triton X-100; 3 = 0,02% Cetyl trimethyl ammonium bromide; 4 = 0,02% sodium deoxycholate; 5 = five freezings and thawings.
Activities: A = natons oxygen/min/ml; B = μmoles guaiacol x 10^2/min/ml; C = μg inorganic phosphorus /30 min/ml; D = mg protein/ml.

creased activity of this enzyme which is not accompanied by any solubilization. This physiological activation seems, therefore, not to be simply due to an unmasking process, but it should imply a kind of conformational rearrangement of the enzyme structure or removal of a specific inhibitor. The decrease in Michaelis constant of the activated enzyme is in keeping with this conclusion.

3. Mechanism of activation.

In the last part of this paper we present results of experiments devised in the attempt to answer to the following question: By which mechanism is the activation of NADPH oxidase triggered, when bacteria or inert material come in contact with leucocytes?

We have previously shown (15,17,26) that the stimulation of respiration and of HMP pathway activity takes place within a few seconds after the addition of bacteria to leucocyte. Since the

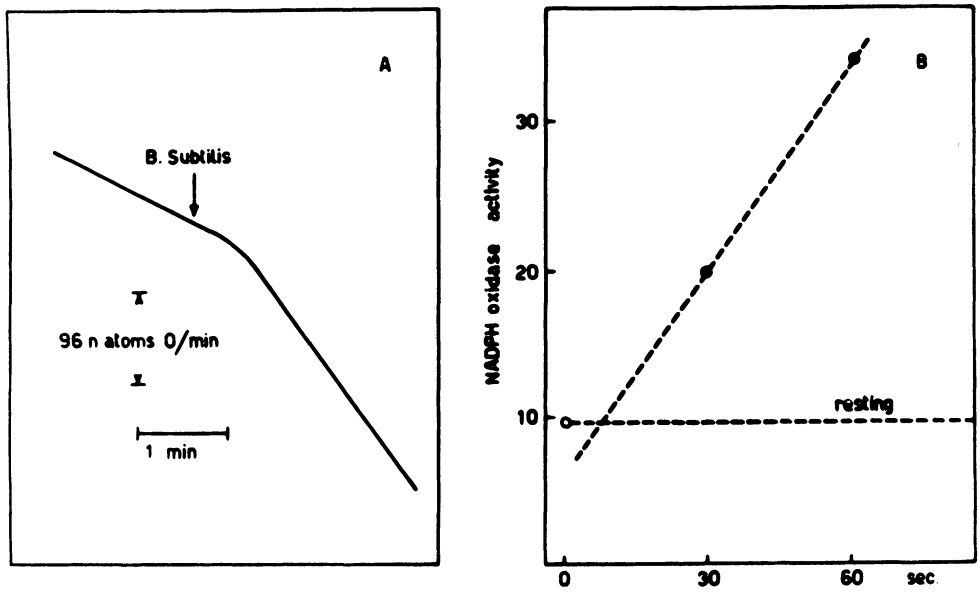

Figure 4 - Early increase of leucocyte respiration and NADPH oxidase activity of granules upon addition of bacteria. Graph A.- Polarographic trace of oxygen uptake by 4.10^7 leucocytes in 2 ml of 0.25M sucrose-bicarbonate, pH7, with 2.5mM $CaCl_2$. Bacteria: leucocyte ratio = 25 : 1. Temperature 37°. Graph B.- Before or after 30 and 60 seconds from the addition of bacteria, leucocytes were frozen in solid CO_2-acetone. After 5 freezings and thawings, they were centrifuged at 100,000 g for 30 minutes. NADPH oxidase activity was assayed both in the pellet and in the supernatant. No activity was detected in the supernatant. Ordinates are nmoles of NADPH oxidized/min/total pellet($2x10^7$cells)

measurement of NADPH oxidase activity has been performed always in granules obtained from leucocytes 3-5 minutes after the addition of bacteria, we have evaluated the time course of NADPH oxidase stimulation by measuring its activity at very short time after the addition of bacteria. As shown in Fig.4 the increase of cell respiration is visible at about 20 seconds from the addition of bacteria (Graph A).

At the same time there is already a marked enhancement of NADPH oxidase activity (Graph B). By linear extrapolation through the activity values at 30 and 60 seconds, one may assume that the ensuing of activation occurs between five and ten seconds after bacteria are put in contact with leucocytes.

The demonstration that the respiratory stimulation induced by addition of particles to leucocytes 1) occurs within a few seconds; 2) is accompanied by a very precocious activation of granule-bound NADPH oxidase; 3) is elicited only when intact leucocytes are used (6); 4) is, at least in some instances, independent of particle ingestion (17,46) and 5) can be reproduced by agents acting on cell membrane, such as surfactants (9, 10,18) and antibodies (11), suggest that some kind of biochemical events, taking place at the surface of the cell, are regulating the activity of granule-bound NADPH oxidase.

In an attempt to clarify what is the role played by the plasma membrane of the leucocyte in the early stage of the activation process we have followed two approaches.

The first approach has been that of producing well defined modifications of leucocyte plasma membrane and analyzing the effect of these chemical alterations on the cell respiration and HMP pathway. The tool used to modify the membrane structure has been phospholipase C (47). Treatment of leucocytes with this enzyme induces a very precocious increase in KCN insensitive respiration and in HMP pathway activity. The stimulating effect is strictly related to the enzymatic hydrolysis of phospholipids. The granules isolated from phospholipase C treated cells show an increase in KCN-insensitive NADPH oxidase, which largely accounts for the burst of respiration (Table V). The treatment with phospholipase C of homogenates or of granules of resting leucocytes do not produce any stimulation of respiration and of NADPH oxidase, the hydrolysis of phospholipids still taking place.

This finding suggests that the activation of NADPH oxidase, and the consequent stimulation of respiration and HMP pathway, are mediated through some changes of the lipid-protein interactions at the external membrane of the leucocyte.

The second approach has been that of using a fluorescent probe of protein and membrane structure (48), 1-anilino-8-naphtalene sulphonate (ANS). When bound to biological membranes this probe gives information about alterations of their structure accompanying biochemical activities, since the fluorescence emission of this fluorochrome is influenced by polarity changes of its microenvironment. Thus it has been possible to read out the dynamic state of membrane as mitochondria, submitochondrial particles, microsomes, erythrocytes and nerves (49-52) through changes in quantum yield and emission spectra of ANS bound to these membraneous materials.

TABLE V

EFFECT OF PHOSPHOLIPASE C ON RESPIRATION AND HMP ACTIVITY OF PMN LEUCOCYTES AND ON NADPH OXIDASE ACTIVITY OF CORRESPONDING GRANULES.

	Control	Phospholipase C
Whole cells (2×10^7)		
a) Oxygen uptake (natoms/min)	15 ± 3	80 ± 8
b) $^{14}CO_2$ from ^{14}C-1-glucose (cpm)	1,892 ± 190	12,034 ± 1500
Granules (from 2×10^7 cells)		
Oxygen uptake (natom/min)	84 ± 12	838 ± 103

Assay media: Whole cells : Isotonic tris buffer pH 7.4 containing 2mM $CaCl_2$, 2.5mM glucose and 2mM KCN.
Granules : see Tab. 1. Oxygen uptake and $^{14}CO_2$ production from ^{14}C-glucose described elsewhere (5). Phospholipase C from Clostridium welchii.

By adding intact PMN leucocytes to a solution of ANS we have noted a marked rise in its fluorescence intensity. Such increase is maintained upon removal of excess ANS by centrifugation, indicating a binding of the dye to hydrophobic regions of the membrane. Binding of ANS to leucocyte does not impair the metabolic responses to phagocytosis.

When polystyrene particles are put in contact with ANS-labeled leucocytes, there is an immediate and irreversible increase of fluorescence intensity, accompanied by a blue-shift in the emission of the chromophore of about 50 nm.

The rate of this interaction is monophasic and very fast, the highest intensity of fluorescence being reached within two seconds. No further change of fluorescence is observed thereafter, for at least 5 minutes.

In order to gain information about the relation of the enhance-

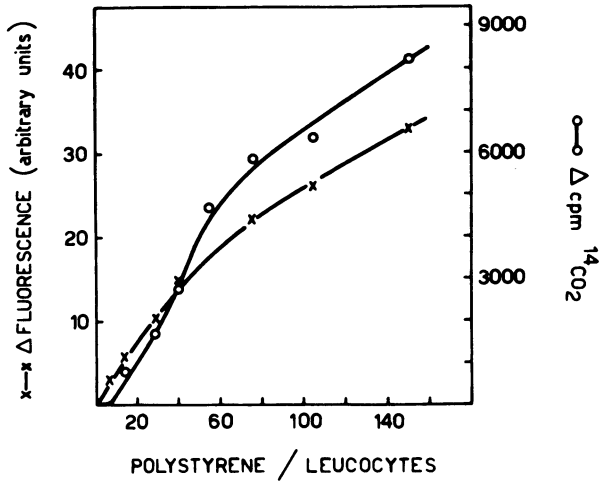

Figure 5 - Increase of fluorescence intensity of ANS bound to PMN leucocytes and of oxidation of 1-^{14}C-glucose upon addition of polystyrene spherules. 1-anilino-8-naphtalene sulfonate (ANS, 40 μM) was added to leucocytes (2 x 10^7 cells/ml) at 30°. After 5 minutes, cells were spun down and resuspended to the original volume. Fluorescence measurements were made at an excitation wavelength of 366 nm, recording the emission between 420 and 3000 nm. $^{14}CO_2$ production from 1-^{14}C-glucose was followed by incubating the leucocytes at 37° for 10 minutes as reported by Rossi and Zatti (5).

ment of fluorescence to the changes in HMP pathway activity induced by interaction between leucocytes and particles, the two events were followed at different polystyrene particles/leucocyte ratios. As shown in Fig.5, by increasing the amount of spherules available to the cells, a parallel enhancement of fluorescence intensity and production of $^{14}CO_2$ from 1-^{14}C-glucose is observed. This suggests that the two processes are somehow linked and dependent on the number of collisions between leucocytes and spherules.

In analogy with what has been claimed for interactions between ANS and other membrane systems, we explain these results as due to conformational transitions of membrane components (with modifications of polarity in regions of the membrane closed to the ANS binding sites), induced by the contact of foreign matter and outer surface of leucocytes.

As previously shown, the activation of respiration of HMP pathway and of granule-bound NADPH oxidase occur within few seconds after the contact leucocyte-bacteria. The apparent change in membrane conformation, as indicated by increase in fluorescence of ANS, takes place within 1-2 seconds, thus preceding any measurable metabolic stimulation.

Furthermore, since the rise in fluorescence of ANS after addition of polystyrene particles to the leucocytes is instantaneous and no further change is thereafter observed, it is not certainly induced by the actual phagocytic process.

Although the precise nature of the conformational changes in the membrane is at yet obscure, we suggest that the correlation between the transitions of structure of the plasma membrane of the phagocyte and the metabolic modifications is provided by the following sequence of events.

1 - Contact between particles and outer surface of leucocytes.
2 - Structural and functional changes of some components of the plasma membrane of leucocytes.
3 - Alteration of the inner layer of plasma membrane, so that either by release of a chemical mediator or by direct interaction between plasma membrane and granules, a signal is transmitted to the NADPH oxidase-containing granules.
4 - Action of the signal on the NADPH oxidase molecule or on some components of the granule membrane, so that either directly or indirectly a rearrangement of the molecular structure of the enzyme is obtained.
5 - Increased oxidation of NADPH by the activated NADPH oxidase.
6 - Increased availability of $NADP^+$ and stimulation of HMP pathway.

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THE BIOCHEMICAL AND ANTIMICROBIAL ACTIVITIES OF THE PHAGOCYTE[1]

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INTRODUCTION

Many factors determine whether a parasite can establish itself successfully in a host and initiate an infectious process. An important mechanism that a host can employ to combat disease and that can be studied, in vitro, at the cellular level is phagocytosis. The importance of this host-defense mechanism has been apparent since the process was first described by Metchnikoff in the latter part of the nineteenth century. However, the mechanism(s) involved in engulfment and destruction of ingested microorganisms have been the subject of much study and controversy. The early literature dealing with phagocytosis has been adequately reviewed (1, 2). These scholarly works concentrate mainly on the physical forces involved with engulfment and also on the different factors that can effect the phagocytic act. Inexplicably little or no studies on the biochemical aspects of phagocytosis were carried out or reported in this early period. Some scattered reports, however, did appear (3). Somewhat later some excellent work was carried out in Nungester's laboratory. Unfortunately, most of this work was published in thesis form only (4, 5). These workers all demonstrated that the phagocytic act was accompanied by biochemical changes; specifically, an increased respiratory activity. Suter and his co-workers in the early 1950's initiated a series of studies concerned with the interaction of tubercle bacilli and guinea pig exudate cells. They described this interaction at the biochemical level (6-8).

[1]This paper is number XXX in a series entitled "The role of the phagocyte in host-parasite interactions."

These papers, apparently, stimulated a number of other workers in different laboratories to systematically investigate the metabolic events accompanying phagocytosis (9-11). All of this work has been carefully reviewed by Karnovsky (12). More recently reviewers from somewhat different vantage points have attempted to correlate the voluminous literature that has erupted in this area in the past decade (13-15).

The purpose of this report is to briefly describe some of the biochemical events that occur during phagocytosis and specifically to relate these events to physiological function.

BIOCHEMICAL ACTIVITIES ACCOMPANYING PHAGOCYTOSIS

The addition of particulate material to guinea pig exudate cells, consisting mainly of polymorphonuclear neutrophilic leukocytes (PMN), results in a number of stimulated metabolic events. Briefly, an immediate and dramatic increase in oxygen consumption and flow of glucose through the hexose monophosphate shunt (HMS) occurs. Also, increased glycolytic activity is noted. Respiratory inhibitors, such as cyanide and antimycin A generally do not inhibit particle uptake. Glycolytic inhibitors, however, inhibit engulfment and generally inhibit the stimulated metabolic activities. These data suggest that the principal energy source for particle entry is glycolysis (9-11).

RELATIONSHIP OF INCREASED METABOLIC ACTIVITIES WITH PHYSIOLOGICAL FUNCTION

It was noted that a number of different situations where the increased oxidative metabolic activities were inhibited, a simultaneous inhibition in bactericidal activity was detected (16). This is particularly evident in the following situations.

a. X-irradiation. PMN collected from guinea pigs x-irradiated with doses as low as 100 r showed defects in phagocytic, bactericidal, and metabolic activities. The maximal inhibition in respiratory, HMS and bactericidal activities were noted 3-5 days post-irradiation. Within approximately 2 weeks all the functions essentially returned to normal (17,18).

b. Anaerobiosis. Under anerobic conditions the stimulated oxidative metabolic activities usually accompanying phagocytosis would be

expected to be minimal. When phagocytosis was permitted to occur in an atmosphere of nitrogen (19), both metabolic and bactericidal activities of the cells were diminished.

c. Phenylbutazone. Additional support for the concept that a relationship exists between oxidative activities during phagocytosis and bactericidal activity was gained by the use of the anti-inflammatory drug phenylbutazone. This drug inhibits glucose-6-phosphate dehydrogenase (G6PDH). G6PDH inhibition results in the simultaneous inhibition of the HMS. Under these conditions complete inhibition of bactericidal activity resulted (20).

d. Chronic granulomatous disease. PMN collected from young children with chronic granulomatous disease (CGD) do not respond to phagocytosis with the increased respiratory and HMS activity generally noted (21). The bactericidal activity of these cells has been found to be significantly decreased. Interestingly,children with this disorder experience repeated bacterial infections with organisms of generally low virulence (22).

e. Acute leukemia. Acute leukemia in children is often accompanied with repeated bacterial infections. We have recently studied both bactericidal and HMS enzyme activities of leukocytes from children with this disease in remission and relapse. Bactericidal activity of these cells was significantly less than those noted from hospitalized control children. The G6PDH of PMN collected from children in relapse had a significantly lower level than that noted in PMN collected from children free of this disorder. PMN isolated from children in remission showed an intermediate level of this activity. These data provide additional support for the role of the HMS involvement in the intracellular bactericidal activity of the phagocyte (23).

MECHANISM BY WHICH OXIDATIVE REACTIONS MAY BE RELATED TO BACTERICIDAL ACTIVITY

From the above it would appear that the increased oxygen uptake and flow of glucose through the HMS noted in phagocytizing cells are related to the bactericidal activities of the cell. The mechanism by which these activities are involved is of interest. Nicotinamide adenine dinucleotide phosphate (NADP) has been reported to be limiting HMS activity in the leukocyte (24). Its regeneration would be critical for the stimulated HMS to be continuously operative in the cell. It has been shown that the reduced nucleotide (NADPH) can be oxidized in the presence of molecular

oxygen by its oxidase yielding H_2O_2 and NADP. It has also been noted that glutathione reductase and peroxidase are present in the cell and that in coupled reactions the reductase too can regenerate NADP from NADPH. Both NADPH oxidase and glutathione reductase have been shown to be stimulated during phagocytosis (25,26).

It was suggested by Quastel et al in 1961 that the H_2O_2 resulting from the stimulated HMS could be involved with the bactericidal activities of the cell (27). Initially, this hypothesis was received with little enthusiam. At approximately the same time, Cohn and Hirsch noted that phagocytosis was accompanied by a loss of granules (lysosomes) (28). This degranulation resulted in the release of a number of different hydrolytic enzymes into the phagocytic vacuole. It was suggested that these agents could be responsible for the killing and degradation of the ingested microorganisms. Somewhat later, Klebanoff reported that lactoperoxidase, H_2O_2 and a halide formed a potent bactericidal system (29). Agner (30) and Schultz (31) have shown that PMN are rich in a granule-associated peroxidase, myeloperoxidase (MPO). These observations led our laboratory to test the granular pellet of the PMN alone and in the presence of H_2O_2 for bactericidal activity. Each component by itself was ineffective as a bactericide at the concentrations employed. The combination of both, however, exhibited significant bactericidal activity. The halide requirement of the systm was satisfied by the chloride ion contained in the suspending medium employed in the experiment (32). The lysed granular pellet was found to contain the bulk of MPO activity of the PMN. At approximately the same time Klebanoff reported that MPO isolated from human PMN could substitute for lactoperoxidase in his bactericidal system (33).

Interestingly, it has been noted that phagocytizing leukocytes have higher H_2O_2 content and peroxidase activity than resting cells. The peroxidase is not only stimulated but also redistributed within the cell. Thus, particle uptake would facilitate MPO-H_2O_2-complex formation (34,35). The physiological significance of this mechanism appears real as it has been shown that patients deficient in MPO are more prone to certain infections (36).

MECHANISM BY WHICH THE PEROXIDASE-H_2O_2-HALIDE SYSTEM FUNCTIONS

This system has been shown to be effective against a number of different microorganisms. In addition to both some gram-positive and

gram-negative bacteria (32), fungi (37) and viruses (38) have also been found to be susceptible. Collaborative studies currently in progress in our laboratory suggest that some strains of Mycoplasma are also susceptible.

The mechanism by which this complex exerts its anti-microbial activity is presently being studied. Klebanoff has suggested that iodination of bacteria may be the lethal bactericidal step (33). However, some question regarding the proposed mechanism must be raised as iodination of bacteria does occur without the expected bactericidal activity (33).

An observation by Zgliczynski et al (39) concerning the MPO activity of PMN isolated from patients with granulocytic leukemia appears pertinent to the elucidation of the mechanism of action of the MPO-H_2O_2-halide complex. These workers demonstrated that a purified MPO preparation from these cells was able to decarboxylate and deaminate amino acids. The reaction product resulting was an aldehyde with one carbon less than the amino acid employed. They further showed that the reaction was H_2O_2 dependent, required chloride ions and had a pH optimum of around 5.0. It should be noted that these requirements are similar to those required for the MPO-H_2O_2-chloride bactericidal system. Further, since aldehydes are potent bactericidal agents, the possibility that the antimicrobial system in the phagocyte was functioning by this mechanism is most attractive. Appropriate substrates for aldehyde formation i.e. amino acids or fatty acids, could be readily supplied by either the ingested bacteria or the phagocyte. It has been found that guinea pig PMN granules, containing MPO, decarboxylate and deaminate both D and L amino acids (40, 41). Decarboxylation of D and L-1-^{14}C-alanine may be seen in Table 1. An attempt to actually measure aldehyde production has been made (42). These results may be noted in Table 2. It is evident that significantly higher levels of aldehyde are recorded when the complete antimicrobial complex is present. The question regarding the substrate source of aldehyde formation is pertinent. It would appear from the results shown in Table 3 that the direct contact of the MPO-H_2O_2-chloride system with bacteria is critical (42). This would suggest that the peroxidative activity is directed mainly at the bacterial surface. The free carboxyl and amino groups that have been reported to be associated with the bacterial cell wall suggest that this hypothesis is plausible.

Indeed Klebanoff has shown an electron micrograph of human leukocytes containing engulfed bacteria which are surrounded by peroxidase positive material (43).

TABLE 1

Decarboxylation of l-^{14}C-D-alanine and l-^{14}C-L-alanine by 20,000 g Granules from Guinea Pig PMN Leukocytes

	H_2O_2	
	-	+
l-^{14}C-D-alanine	0.54[a]	144.21
l-^{14}C-L-alanine	0.49	146.18

[a] mμmoles $^{14}CO_2$/flask

Each flask contained 5.4 μmoles of radioactive amino acid (0.055 μCi/ moles); 2 x 10^{-4} M H_2O_2 where indicated; 0.03 guaiacol units of peroxidase containing granules and KRPM, pH 5.5, to 3.0 ml volume. $^{14}CO_2$ was trapped in 0.2 ml of 20% KOH in the center well. Reactions were run for 30 minutes at 37°C in a shaking water bath (90 strokes/min) and were terminated by addition of 0.2 ml of 30% TCA from the side arm. See text for complete details. (Infection and Immunity, submitted for publication)

TABLE 2

Estimation of Aldehydes in the MPO-H_2O_2-Chloride Bactericidal System

Supplements	Organisms/ml[a]	mμmoles of aldehydes[b]
E. coli	1.2 x 10^4	6.3 ± 2.3
E. coli + MPO	1.2 x 10^4	3.0 ± 1.4
E. coli + H_2O_2	1.3 x 10^4	1.0 ± 1.0
E. coli + MPO + H_2O_2	$<5.0 \times 10^0$	22.9 ± 5.6
E. coli + heated MPO[c]	1.2 x 10^4	5.8 ± 1.9
E. coli + heated MPO + H_2O_2	1.2 x 10^4	5.8 ± 1.9

[a] Incubations were carried out for 5 minutes at 37°C. The bactericidal system contained where indicated, H_2O_2, 0.4 μmoles; MPO (guinea pig PMN granules), 0.016 guaiacol units; 8 x 10^4 E. coli and KRP B, pH 5.5, to 8.0 ml.

[b] Total measurable aldehyde per reaction system. Mean ± standard error of the mean given in each case.

[c] Heated in a boiling water bath for 30 minutes.

See text for additional details. (Infection and Immunity, 2:414-418, 1970).

TABLE 3

Bactericidal Activity of the MPO-H_2O_2-Chloride System in the Presence and Absence of Direct Bacterial Contact with MPO

	Number of viable organisms per reaction tube
1. Direct contact[a]	
E. coli	2.0×10^4
E. coli + MPO	2.1×10^4
E. coli + H_2O_2	1.8×10^4
E. coli + H_2O_2 + MPO	2.2×10^1
2. No Direct Contact[b]	1.7×10^4

[a] Bactericidal system containing 0.012 guaiacol units of MPO, 0.4 μmole H_2O_2, 2×10^4 E. coli and KRPB, pH 5.5 to 8.0 ml as indicated above.
[b] 2×10^4 E. coli placed inside a dialysis bag containing 0.1 μmole H_2O_2 and KRPB, pH 5.5 to a volume of 2.0 ml. Dialysis bag was placed in 6.0 ml of pH 5.5 KRPB containing 0.012 guaiacol units of MPO and 0.3 μmoles of H_2O_2. The complete reaction system was incubated at 37°C for 30 minutes. Viability was monitered by sampling the contents in the dialysis bags. (Infection and Immunity, 2:414-418, 1970)

This antimicrobial system (MPO-H_2O_2-halide) has been reported to function with either bromide, chloride, or iodide as the halide component (44). However, by substituting horseradish peroxidase (HPO) for MPO, the system is only bactericidal when iodide is the halide (42). Interestingly, HPO does not mediate amino acid decarboxylation with either halide, and MPO containing granules show decarboxylation activity only when chloride is the participating halide (41, 42) These results may be seen in Table 4. The possible significance of these observations was provided by the use of the amino acid taurine. This amino acid competitively inhibits amino acid decarboxylation and deamination mediated by purified MPO (39). It also inhibits peroxidase-associated bactericidal activity in a chloride medium (40, 45). On the other hand, taurine did not inhibit the bactericidal activity of the peroxidase H_2O_2-iodide system (40). These results may be seen in Table 5. It would appear that these two different halides function in the peroxidase mediated bactericidal activity by two different mechanisms. Chloride appears to activate the MPO-H2O2-mediated deamination and decarboxylation

TABLE 4

The Effect of Different Halides on Peroxidase Mediated Decarboxylation and Bactericidal Activity

Supplements	Percent bacteria killed	Decarboxylation[b] DPM
MPO + Cl + H_2O_2	99	24,000
HPO + Cl + H_2O_2	0	120
MPO + I + H_2O_2	99	80
HPO + I + H_2O_2	99	52

Incubations for both (a) and (b) were at 37C for 30 minutes. Results of complete system for each set of experiments are presented. In the absence of any of the components,no significant bactericidal or decarboxylation activity was noted.

[a] Reaction mixture contained 2 x 10^4 E. coli and the supplements as indicated below: H_2O_2, 0.1 μmoles; MPO, 0.003 guaiacol units or HPO, 0.018 guaiacol units; KI, 0.2 μmoles or NaCl 200 μmoles. 0.1 M halide free phosphate buffer, pH 5.5 to a total volume of 2.0 ml.

[b] Reaction mixture contained 0.24 guaiacol units of HPO or 0.03 guaiacol units of MPO; 0.3 μmoles of KI or 300 μmoles NaCl, H_2O_2; 0.3 μmoles; and l-^{14}C-L-alanine (specific activity 0.055 μCi/ μmoles) and 0.1 M halide free phosphate buffer, pH 5.5 to 3.0 ml.

See text for details. (Infection and Immunity, 2:414-418, 1970)

TABLE 5

The Effect of Tuarine on Amino Acid Decarboxylation and Bactericidal Activity of 20,000 g Granules in the Presence of Chloride or Iodide

Additions	NaCl		NaI	
	$^{14}CO_2$ [a]	Survivors [b]	$^{14}CO_2$	Survivors
0	0.76	1.20×10^4	0.45	1.26×10^4
H_2O_2	0.82	1.19×10^4	0.32	1.08×10^4
Halide	1.06	1.10×10^4	0.31	1.23×10^4
Halide + H_2O_2	188.42	2.50×10^0	0.42	2.50×10^0
Halide + H_2O_2 + Taurine 4×10^{-5} M	188.40	2.50×10^0	0.37	2.50×10^0
Halide + H_2O_2 + Taurine 4×10^{-4} M	118.90	4.39×10^2	0.35	2.50×10^0
Halide + H_2O_2 + Taurine 4×10^{-3} M	9.00	0.68×10^4	0.45	2.50×10^0

[a] Each flask contained 0.03 guaiacol units of 20,000 g granules, 0.6 μmoles of H_2O_2, 450 μ moles of NaCl, 0.15 μmoles of NaI and taurine as indicated; 5.4 μmoles of l-^{14}C-L-alanine (specific activity 0.055 μCi/μmole) and 0.1 M, pH 5.5, phosphate buffer to 3.0 ml total volume. Incubation was for 30 minutes. All values represent mμmoles/flask. See text for additional details.

[b] Each tube contained 0.03 guaiacol units of granules, 1.2×10^4 E. coli/ml and where indicated 0.3 moles of H_2O_2, 900 μmoles of NaCl, 0.30 μmoles of NaI taurine and 0.1 M, pH 5.5, phosphate buffer to 6.0 ml final volume. Reactions were run for 30 minutes. See text for complete details. (Infection and Immunity, submitted for publication).

of substrates whereas iodide may be exerting its bactericidal effect by some other mechanism related to its involvement in the peroxidase reaction.

Some insight regarding which of the halides may be functioning at which sites in vivo is gained by considering the concentrations of each halide required for activity. Concentrations of iodide required for in vitro activity are generally not attainable in vivo except in secretions at sites of iodide concentration, such as the thyroid and salivary glands. Concentrations of iodide necessary for optimal antimicrobial activity are at least 600% higher than circulating blood iodine levels. The chloride concentrations that are effective in vitro are in the range of serum chloride levels (40, 42, 44).

The requirement of chloride for amino acid decarboxylation and the effect of this halide on guaiacol oxidation of MPO is of interest. It has been noted that MPO can either decarboxylate and deaminate appropriate substrates or it can oxidize guaiacol type substrates. In the presence of chloride and low pH, decarboxylation and deamination are favored. At pH 5.5, in the presence of chloride, guaiacol oxidation is over 90% inhibited. At pH 7.4, no inhibition of guaiacol oxidation is detected. Since the intracellular chloride concentration is less than that of the extracellular fluid and the intracellular pH is near 7.0, this enzyme would not be expected to produce significant quantities of aldehyde when the cell is not engaged in phagocytosis. During phagocytosis myeloperoxidase,as well as (35) extracellular fluid rich in chloride,enter into the phagolysosome. The pH of this vacuole has been shown to be acidic (46). These changes would tend to favor the decarboxylation and deamination reactions. It would appear that the extracellular chloride and lowered pH could act to "trigger" the decarboxylation and deamination reactions and "shutdown" guaiacol oxidation by MPO (40).

OTHER ANTIMICROBIAL FACTORS

It must be pointed out and emphasized that the antimicrobial system defined above should certainly not be expected to be the only system in the cell that will kill the many different microorganisms that the phagocyte may be faced with. Phagocytin (47), cationic proteins (48), leukin (49) are other agents which have also been described.

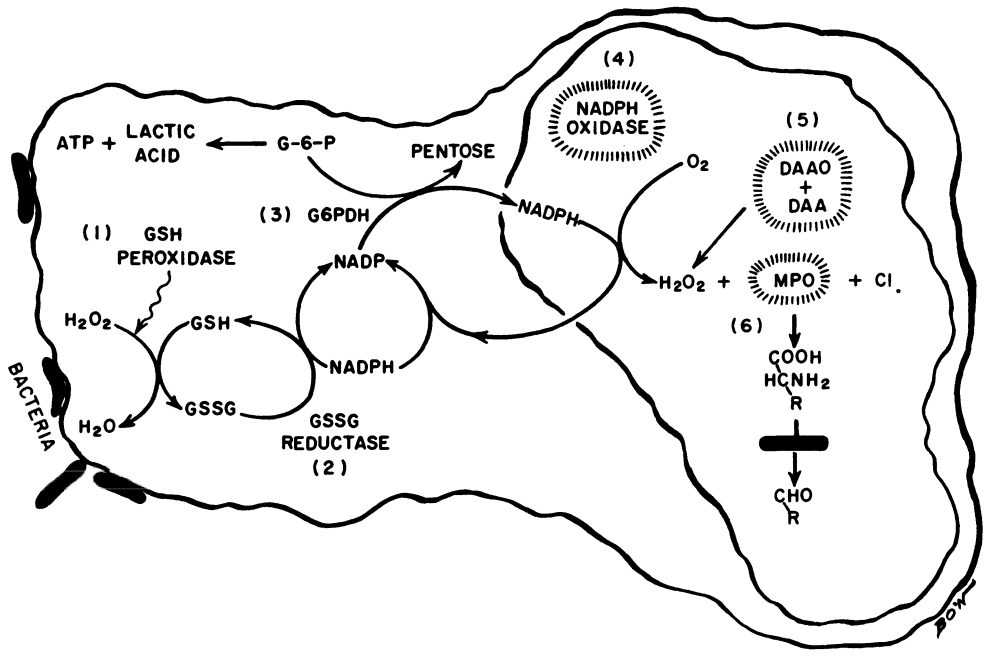

FIGURE 1

A PROPOSED MECHANISM RELATING PHAGOCYTIC, BACTERICIDAL & METABOLIC ACTIVITIES IN PMN DURING PHAGOCYTOSIS

SUMMARY

Figure 1 diagramatically presents some of the biochemical events that occur during phagocytosis. The possible relationship of these events to bactericidal activity is shown. Glycolytic activity appears related to engulfment. Oxidative metabolic activity on the other hand appears related to the bactericidal activity of the cell. The increased H_2O_2 content and stimulated MPO activity of the phagocytizing cell have been shown to be involved in the production of aldehydes. These compounds have been postulated to be the actual bactericidal agents resulting from the increased oxidative activity associated with phagocytosis.

ACKNOWLEDGMENTS

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ANTIMACROPHAGE SERUM: ITS EFFECT ON THE IMMUNOLOGICAL COMPETENCE AND LYMPHOID TISSUES OF THE RAT

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While macrophages are thought to play a role in immune responses, their precise function has not been clearly defined. It has been suggested that macrophages "trap" antigens during the primary response, process the ingested material (1) and communicate "immunological information" to lymphocytes which initiate antibody formation (2). Macrophages may also destroy target cells, suggesting they have a role in the efferent as well as the afferent arm of the immune response (3). Since antisera raised against macrophages may further delineate the role of the cells in the immune response, this study was undertaken to determine the effects of antimacrophage serum (AMS) on lymphoid tissues and to compare these effects and the effect of AMS on cellular immunological competence with those observed after antilymphocytic serum treatment.

MATERIALS AND METHODS

Animals

Young adult Lewis rats and the F_1 hybrids derived from the Lewis and Brown Norway parental strains (F_1 [Lew x BN] hybrids) were utilized throughout this study. At the time of injection or cell collection the animals weighed between 125-175 grams. The antisera were raised in one kilogram, white, male New Zealand rabbits.

Antimacrophage Serum

The antimacrophage serum was prepared with macrophages obtained by injecting Lewis rats intraperitoneally with 1 cc of a 10% saline solution of Higgins India ink. Forty-eight to 72 hours later, the peritoneal cavity was washed with 10 ml of Eagle's medium containing 10 units/ml of heparin. After isolating the cells by centrifugation, they were cultured for 3-6 hours at 37°C in Rose chambers containing Eagle's medium, heat-inactivated, normal rabbit serum (NRS) and antibiotics. This allowed the phagocytic cells to attach to the glass coverslips. The medium was then decanted and the chambers washed with Hank's Balanced Salt Solution (Hank's BSS) to remove nonadherent cells. The chambers were refilled with a solution of calcium and magnesium-free EDTA and allowed to stand for 10-15 min (4). Subsequent repeated agitation and washings resulted in the detachment of the macrophages from the coverslips. Differential counts based upon morphology and the phagocytosis of India ink revealed that 90-95% of the cells were macrophages (Fig. 1). Fifteen to 25 million cells of this macrophage-rich solution were then suspended in 2 cc of a solution containing equal volumes of Hank's BSS and Freund's complete adjuvant for the first two immunizations and Freund's incomplete adjuvant for the subsequent five injections. One week following biweekly intramuscular injections, the rabbits were exsanguinated

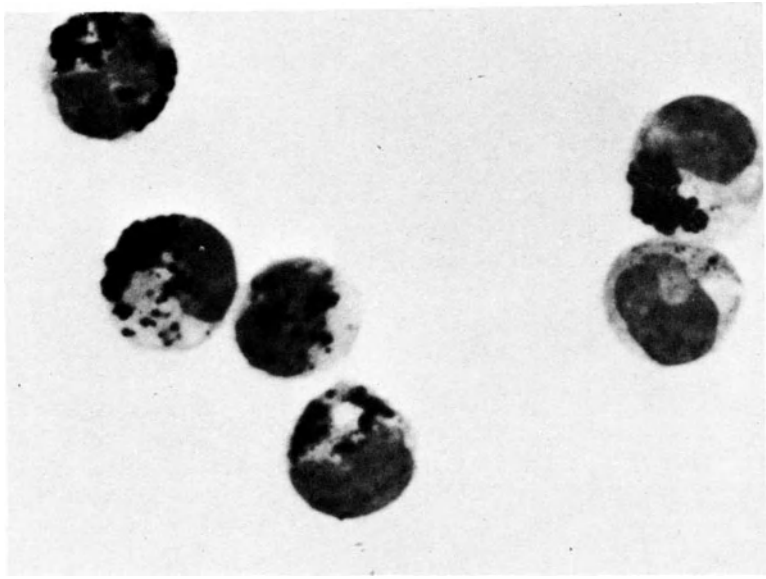


Fig. 1. Phagocytic mononuclear cells containing India ink which were obtained from the peritoneal cavity and isolated by their ability to attach to glass in culture.

by cardiac puncture and the serum separated by centrifugation. The complement was then deactivated by heating the serum for 30 min at 56°C, and the hemagglutinating antibodies were removed by absorbing the serum three times with erythrocytes. The antisera were stored at -4°C until injected i.p. in 1 cc doses. Serum from a rabbit injected with Freund's adjuvant was prepared as a control in a manner identical to that described above for AMS production without the addition of cells.

Cell Collection and Cell Counts

From the cell counts performed with routine laboratory hemocytometers and from differential counts performed on smears, the absolute numbers of the various cell types in the blood were determined at intervals before and after the intraperitoneal administration of AMS. The cell number in the thoracic duct lymph of the animals was determined immediately after establishing a cervical fistula according to the method of Reinhardt and Li (5). The animals were then hydrated with up to 4 cc of a 5% glucose in saline solution to encourage lymph flow. Subsequently, the cells were collected for the mixed lymphocyte (MLR) and normal lymphocyte transfer (NLT) reactions. Suspensions of cells for the MLR and the NLT were also obtained from the lymph node, spleen and thymus by mincing these organs in Eagle's medium.

Histology

The lymph nodes, livers, spleens and thymuses were carefully removed at 2, 4 and 6 hours following AMS or NRS treatment. After fixation of small (2 x 2 mm) sections of the tissues in phosphate-buffered OsO_4 for 1-1/2 hours, they were dehydrated in ethyl alcohol and washed twice with propylene oxide. The tissues were then embedded in Epon epoxy resin, sectioned on a Porter Blum II Microtome and stained 3 min with uranyl acetate and Reynold's lead stain before viewing in an RCA 2A or 2C electron microscope.

The 1 μ sections for light microscopy were prepared in the same way as for electron microscopy except that they were stained with the basic fuchsin and methylene blue stain of Huber et al. (6).

Normal Lymphocyte Transfer Reaction

NLT reactions were initiated with suspensions of lymphoid cells from the thoracic duct lymph, spleens, lymph nodes or thymuses of NRS- or AMS-treated Lewis rats using the method of Ford (7). This entailed injecting parental Lewis cells intradermally into the ventral abdominal wall of F_1 (Lew x BN) hybrids. Five days later 1 cc of a solution containing 1% methylene blue was injected intravenously into the F_1 rats. The dye accumulated at

the sites of the cellular injection over the next 6-8 hours and delineated the reactions. The abdominal skin was then removed and the maximum diameters of the lesions were measured. The presence of reactions was interpreted as meaning that the injected Lewis cells were immunologically competent as evidenced by their reaction to the Brown Norway component of the F_1 hybrid (8).

Mixed Lymphocyte Reaction

Aliquots of the thoracic duct cells used to initiate the normal lymphocyte transfer reactions were also used to prepare the MLR according to the method of Schwarz (9). Under these conditions, some cells were cultured alone without stimulation (nonmixed control) while other cells were nonspecifically stimulated with pokeweed mitogen (nonspecific transformation control). The experimental mixed cultures contained equal numbers of Lewis and F_1 (Lew x BN) hybrid donor cells. In the experimental cultures, if the Lewis small lymphocytes were immunologically competent they would undergo transformation to large, immature-appearing blasts in response to the BN component of the F_1 hybrid. This transformation was quantitated by exposing the cultures, after 72 hours, to 20 μc/ml of H^3-thymidine (6.7 c/mM). Eight hours later, the cultures were sacrificed and the H^3-thymidine uptake was determined by liquid scintillation assay.

RESULTS

Peripheral Blood

A single i.p. injection of AMS had a marked effect on the peripheral leukocyte population as evidenced in Fig. 2. Within the first 2 hours after treatment the absolute number of leukocytes fell 40-45% below the original counts. By 4 hours the counts were 70-75% below control levels. The lowered blood leukocyte counts persisted for nearly 3 days before a rise to normal levels occurred. In contrast to the change induced by AMS, NRS produced initially a transient increase in the leukocytes.

Examination of the blood compartment revealed alterations in the absolute number of monocytes, neutrophils, and lymphocytes subsequent to AMS injection. As seen in Fig. 3, the depression in leukocytes can be partially accounted for by a total loss of monocytes by 2 hours post-AMS. This decrease of monocytes persisted for a period of 2-3 days with a gradual restoration of normal levels. In the same 2-hour period, virtually all of the neutrophils disappeared (Fig. 4). This drastic reduction of cells persisted for 2 days with a gradual increase by 4 days. A decrease of lymphocytes also contributed to the total reduction in leukocytes. Since by 4 hours the lymphocytes levels were only 65%

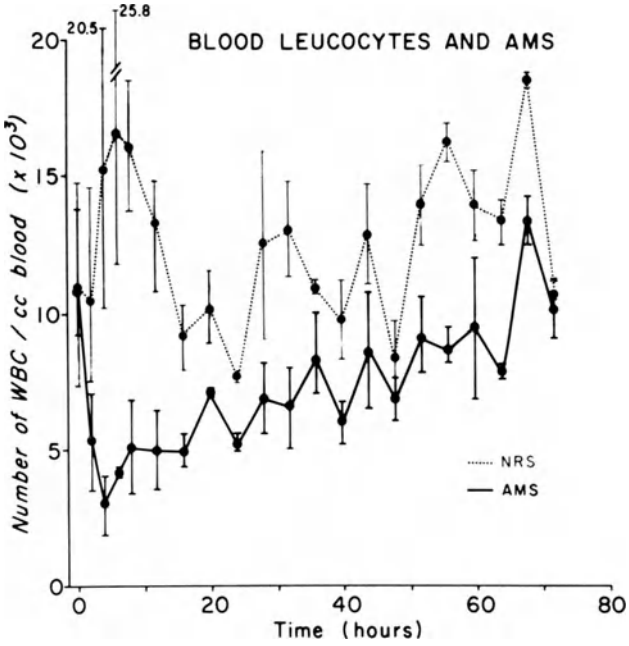


Fig. 2. The effect of a single injection of AMS or NRS on the absolute number of peripheral blood leukocytes. Note the significant reduction as soon as 2 hours post-AMS. The ranges of counts observed are indicated by the bars.

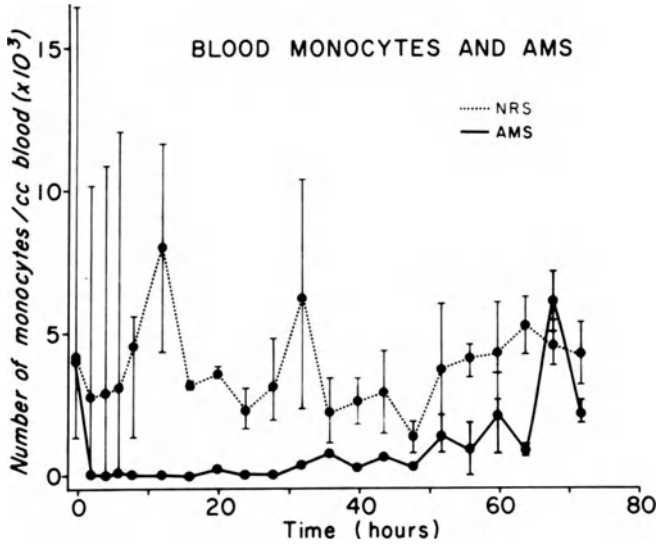


Fig. 3. The effect of a single injection of AMS or NRS on the absolute numbers of monocytes in the peripheral blood.

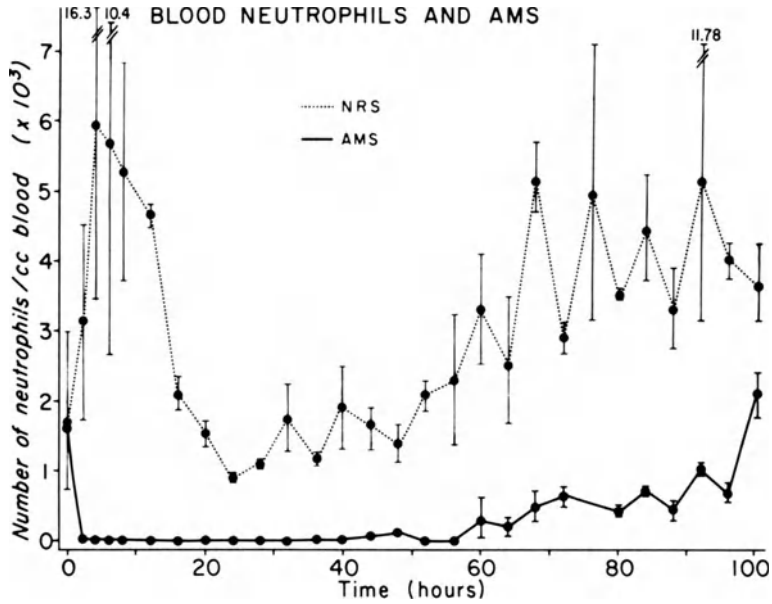


Fig. 4. The effect of a single injection of AMS or NRS on the absolute numbers of neutrophils in the peripheral blood.

of pretreatment values. This reduction remained only slightly lower than for the NRS-treated animals for approximately 3 days. The effect of serum from rabbits injected with only Freund's adjuvant on the lymphoid tissues was not dissimilar to that of NRS. The number of lymphocytes in the thoracic ducts of AMS-treated donors 6 hours postinjection was 53 x 10^6/cc as compared to 88 x 10^6/cc in the NRS-treated controls.

Tissues

The reduced numbers of peripheral blood monocytes, neutrophils, and lymphocytes after AMS treatment was reflected in changes observed in the lymphoid tissues of the treated animals. In the liver, phagocytosis of leukocytes was readily apparent by 2 hours and persisted through 6 hours (Figs. 5, 6). At later intervals, degradation rendered many of the cells nonidentifiable, although at 2 hours, large numbers of neutrophils were recognizable in normal-appearing Kupffer cells. Monocytes and lymphocytes were recognized much less frequently. The electron microscopic section (Fig. 6) shows a vacuole within a Kupffer cell which contains an ingested neutrophil. Comparable results to those of the liver were observed in the spleen (Fig. 7). In this case, intense phagocytosis was evident 2 hours after treatment and increasing

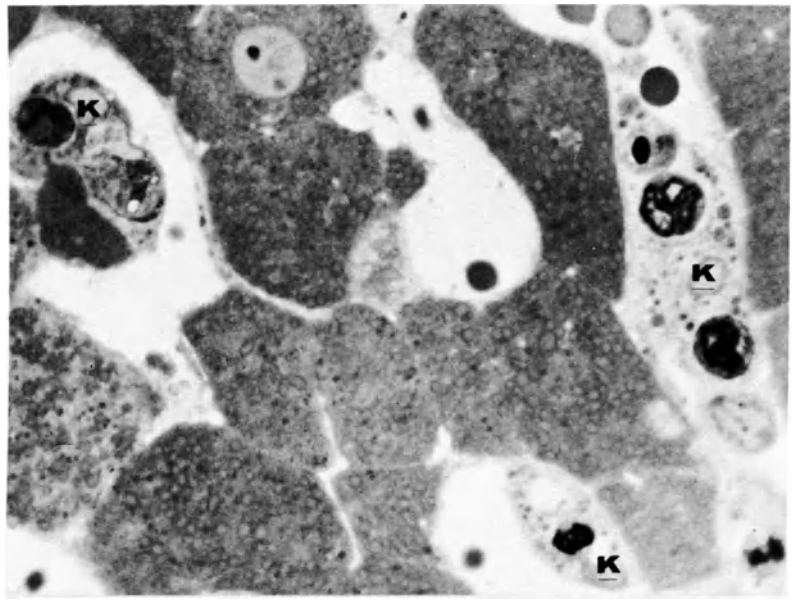


Fig. 5. Photomicrograph of a 1 μ section of liver 4 hours after an i.p. injection of AMS. Phagocytosis and degradation of several leukocytes, many no longer recognizable, by Kupffer cells (K) are shown.

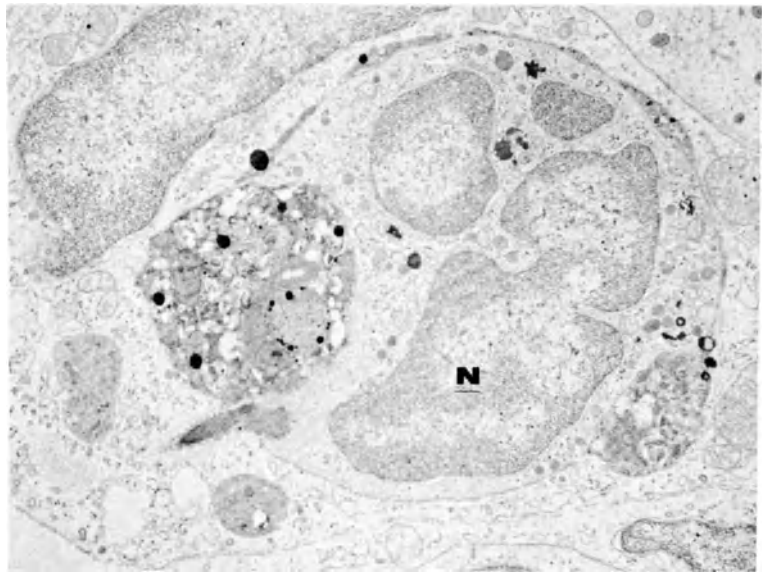


Fig. 6. Electron micrograph of a Kupffer cell containing an ingested neutrophil (N) within a vacuole 6 hours post-AMS injection. X 7000.

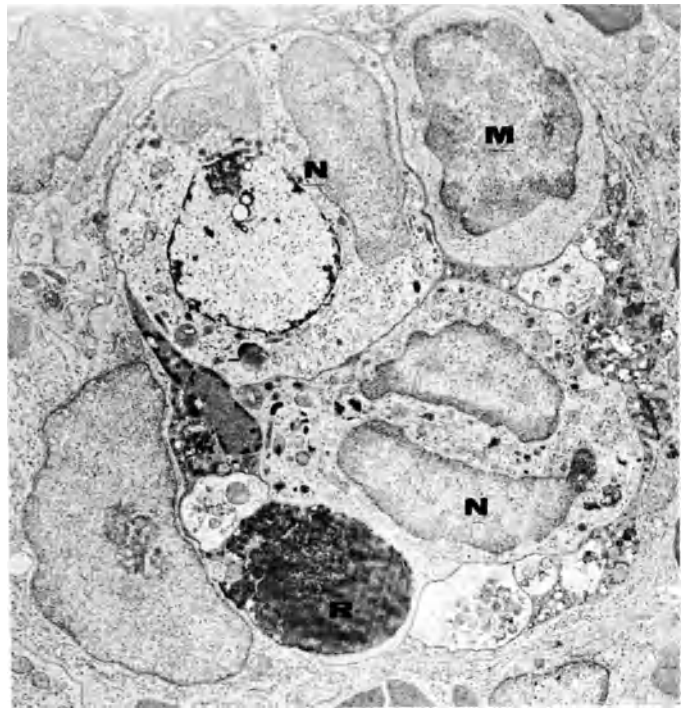


Fig. 7. Electron micrograph of a splenic macrophage which has engulfed 2 neutrophils (N), a mononuclear cell (M), and also contains some red cell debri (R). X 4000.

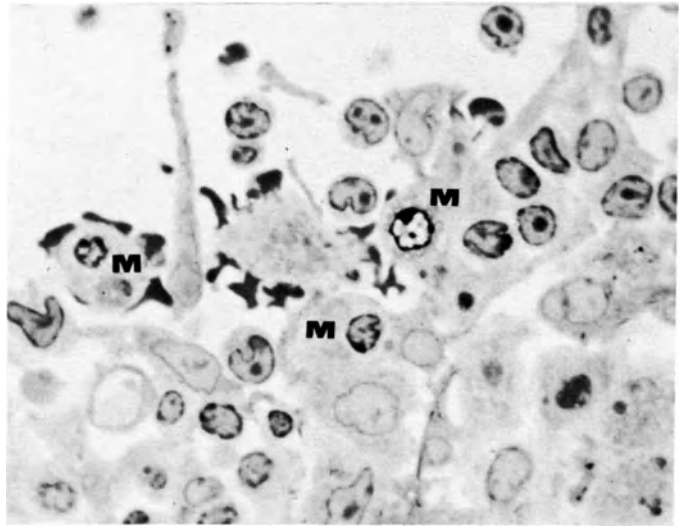
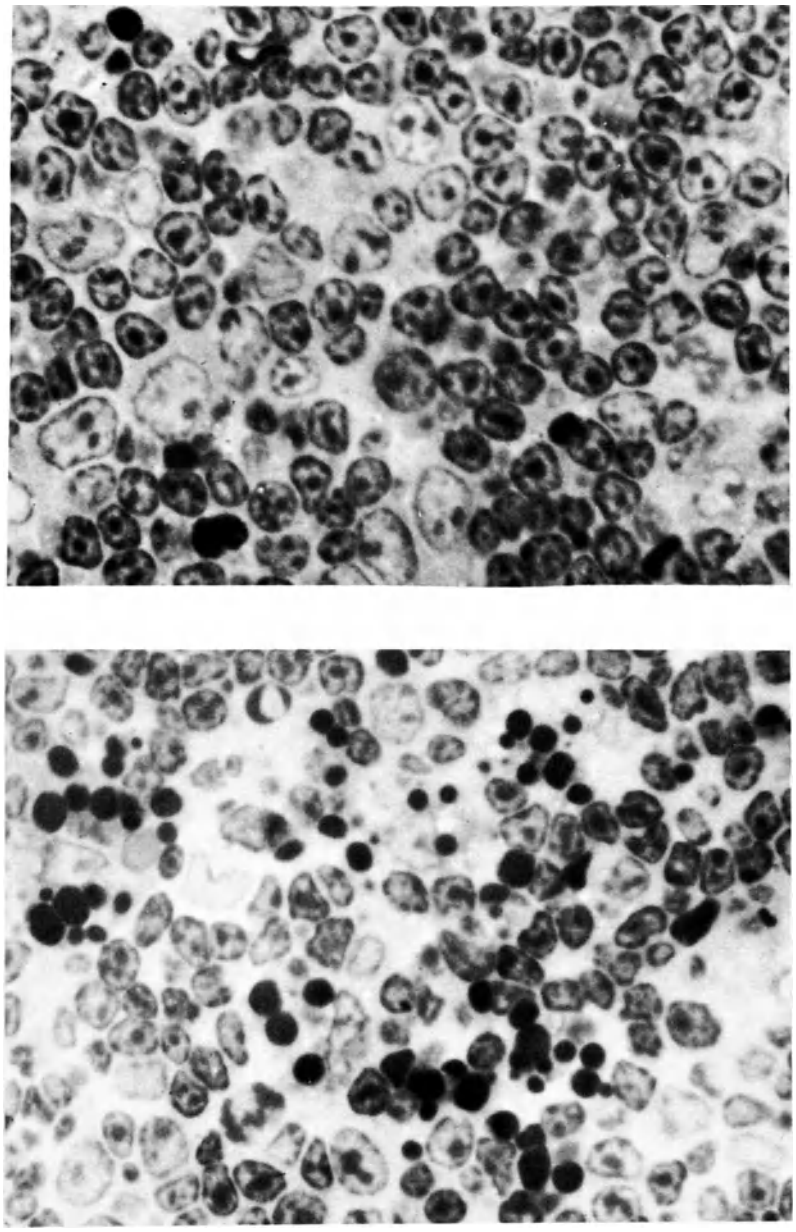


Fig. 8. A section of a cortical lymph sinus of the mesenteric node displaying lymphocytes within macrophages (M) and red cell rosette formation 6 hours after AMS injection.



Figs. 9 and 10. Photomicrographs of the cortical region of the thymus. Fig. 9 (top) is from an NRS control animal. Fig. 10 (bottom) is from the AMS-treated rat, 4 hours postinjection. Note the increased pyknosis of the lymphocyte nuclei.

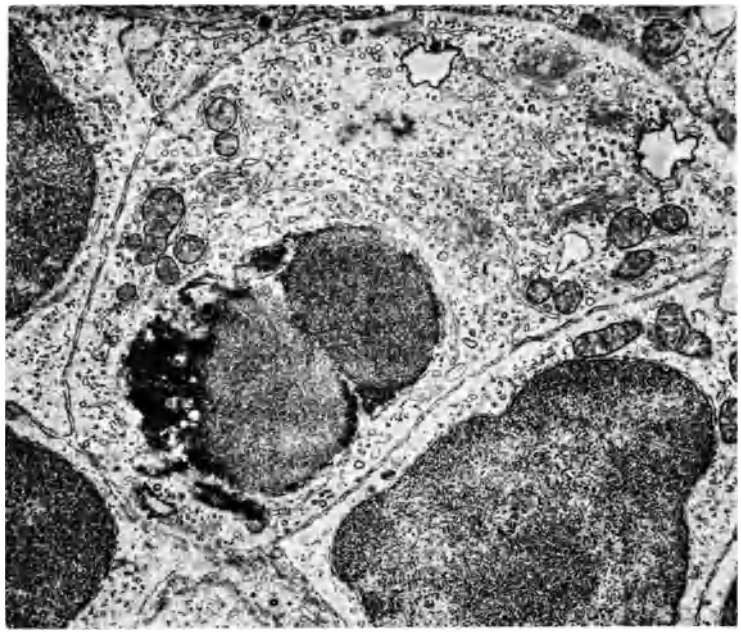


Fig. 11. Electron micrograph of a phagocytic reticular cell in the thymus. Two lymphocytes with pyknotic nuclei are contained within a vacuole. X 7000.

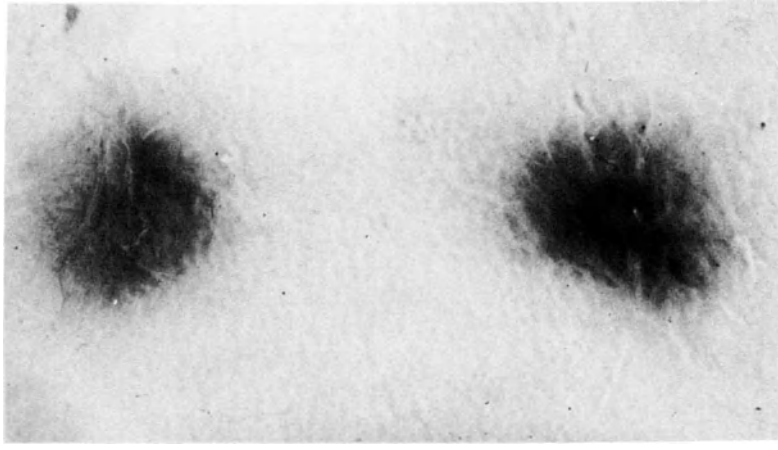


Fig. 12. Photomicrograph of NLT reactions. The reactions were induced by cells from an NRS-treated donor (left) and an AMS-treated animal (right).

Effect of AMS on the MLR	
Culture	CCPM x 10^3
NRS	4.46 ± .69
AMS	5.14 ± 2.2

Fig. 13. The effect of AMS on blast transformation in the MLR using H^3-thymidine uptake as an assay. CCPM x 10^3, thousands of corrected counts (for background) per minute.

cellular destruction was noted during the succeeding 4 hours. By 6 hours, red cell destruction was also evident in the red pulp, while the white pulp showed no significant alterations.

Lymph node histology, although not markedly abnormal, revealed some lymphocyte and red blood cell phagocytosis as well as red cell rosette formation (Fig. 8). The mast cells were increasingly prominent in the medullary region of the node after AMS giving the impression of being increased in numbers. In the NRS-treated controls, the only change observed was red blood cell infiltration of the sinusoids. In the thymus glands, an increased amount of cellular pyknosis was evident 2 hours after AMS. The increased pyknosis, contrasted in Figs. 9 and 10 with the limited pyknosis of the NRS thymus, represented degenerating lymphocytes, some of which had been engulfed by the phagocytic cells of the thymus (Fig. 11).

Normal Lymphocyte Transfer Reaction

Antimacrophage serum had no measurable influence on the initiation of NLT reactions by cells from the lymphoid tissue. As demonstrated in Fig. 12, thoracic duct cells from NRS donors produced lesions 8-10 mm in diameter when injected intradermally into the F_1 hybrids. Cells from AMS-treated animals produced nearly identical lesions, indicating no diminution in the immunological competence of these cells.

Mixed Lymphocyte Reaction

The *in vitro* MLR results paralleled closely the *in vivo* NLT reaction findings. Treatment of the animals with AMS did not reduce the transformation of thoracic duct small lymphocytes (Fig. 13). In addition, cells obtained from spleen, thymus and lymph node transformed normally in the presence of the F_1 hybrid cells.

DISCUSSION

It has been reported that AMS prepared by various methods reacts in a relatively specific fashion against macrophages *in vitro*, producing phagocytic depression (10, 11, 12, 13), inhibition of attachment, and agglutination and degeneration of the macrophages (14, 10, 15, 11). Because these observations imply changes in the macrophages' membranes, it has been suggested that the site of action of the AMS may be on the cell membrane (12).

Whereas AMS appears to have an *in vitro* specificity for macrophages, the *in vivo* effects of AMS are not understood. In contrast to antilymphocytic serum which selectively destroys long-lived small lymphocytes *in vivo* (16; Perkins, unpublished observations), AMS appeared to be nonspecific in that monocytes, lymphocytes and neutrophils were phagocytized. Some authors have reported no significant change in peripheral blood leucocyte numbers of the AMS-treated animals, although Dyminski and Argyris (17) found depressions of mononuclear cells using both AMS and ALS, and Heise and Weiser (18) induced a decrease in lymphocytes and neutrophils with AMS. Repeated injections of the antiserum have also been reported to cause a fall in the white blood cells with a virtual disappearance of neutrophils (15).

Whether the nonspecific phagocytosis of leucocytes observed in this study resulted from antibody raised against contaminating lymphocytes and neutrophils in the immunization suspensions is not known. It may also be possible that macrophage antibodies cross react with the other cell types or that the antiserum causes a nonspecific alteration in the phagocytic capacity of the tissue macrophages. It is interesting in this regard that the histological changes could not be altered by absorbing the AMS with either a suspension of macrophages, neutrophils or lymphocytes. Moreover, Simpson has reported that antineutrophil serum does *not* cross react with macrophages ('70, unpublished observations).

Not only were the histological changes after AMS dissimilar to those observed with ALS, but the effects of these antisera on the initiation of immune responses were also different. In the case of ALS, cells from a treated donor were incapable of producing mixed lymphocyte reactions or normal lymphocyte transfer reactions (19, 20). In contrast, the cells from AMS-treated rats initiated these reactions with an intensity equal to that of cells from an NRS-treated control donor. While it may be suggested that these two reactions do not test macrophage involvement in the immune response, evidence is growing that for the MLR to occur, macrophages are required (21, 22). Supporting this concept is the observation of Jehn *et al.* (23) that AMS will inhibit blast transformation of previously sensitized lymphocytes.

These results are in accord with reports that AMS does not alter antibody formation to certain antigen (15, 13, 10) or suppress previously induced states of delayed hypersensitivity (18). There are, however, some reports suggesting that AMS may act as an immunosuppressant (14, 24, 17, 25). This discrepancy may result from the different methods used to prepare the antiserum.

SUMMARY

This study has demonstrated that when rabbit, antirat macrophage serum was administered to rats, there was a reduction in the number of monocytes, neutrophils and lymphocytes of the peripheral blood. While the reduction in peripheral leucocytes was occurring, phagocytosis of these cells was observed in the livers, lymph nodes and spleens of the treated animals. In addition, increased pyknosis was observed in the thymuses as was engulfment of the pyknotic nuclei. Although the changes in the lymphoid tissues were extensive, there was no alteration in the immunological competence of the cells from lymphoid organs as evidenced by the ability to initiate normal lymphocyte transfer reactions in vivo or the mixed lymphocyte reactions in vitro.

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MACROPHAGE REVERSAL OF ANTI-LYMPHOCYTE SERUM INDUCED IMMUNOSUPPRESSION*

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The development of organ and tissue transplantation as a relatively common surgical procedure has necessitated the employment of immunosuppressive drug therapy in order to eliminate or delay the onset of immunologically-mediated tissue rejection mechanisms by the recipient (1). A wide variety of immunosuppressives, including antimetabolites, e.g., the purine analogs; alkylating agents, e.g., cyclophosphamide; and specific anticellular sera, e.g., antilymphocyte serum (ALS), have been employed. Unfortunately, a common side effect of nearly all immunosuppressive therapy is the development of post-transplant infections and tumor metastases (2-4). Indeed, Berenbaum (2) reported that up to 30% of renal transplantation failures have been associated with infection, while Starzl and Marchioro (3) have found 50% or more of hepatic transplantation failures to be associated with infections. Similarly, Wilson (4) has claimed that patients subjected to immunosuppressive therapy constitute a high risk group in terms of increased incidence of neoplasia.

Early investigators suggested that the development of infection following immunosuppression might be attributed to the leukopenia which develops (5,6). However, Kerby and Martin (7) could find no correlation between leukopenia and the removal and destruction of bacteria from the blood stream by the splanchnic organs. Thus, the determinant of increased susceptibility to infection following immunosuppressive therapy remains undefined.

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Since the macrophage serves as the "first-line of defense" against bacterial invasion, it is possible that some, or all, of the immunosuppressives currently in use induce alterations of the fixed macrophage populations. Such alterations in this host-defense system may result in decreased removal and destruction of bacteria as well as neoplastic cells.

One immunosuppressive agent which is being used with increasing frequency in transplantation is ALS. While many studies have attempted to elucidate the mode of action of ALS (8), there remains little agreement as to the mechanisms involved. The present study was undertaken to define the influence of ALS upon phagocytosis in order to gain an insight into the mechanisms which lead to a predisposition of ALS-treated individuals towards infections and neoplasia. Furthermore, since the macrophage constitutes the afferent limb of the immune reflex arc, studies were also designed to determine the degree to which the macrophage might participate in ALS-induced immunosuppression.

MATERIALS AND METHODS

In the preparation of ALS, adult rabbits received four weekly intraperitoneal injections of 2×10^8 rat (Sprague-Dawley) thymocytes suspended in Tyrode's solution. Ten days following the last injection of thymocytes the rabbits were bled from the marginal ear vein and the serum was heat-inactivated at 56° for 45 min. and adsorbed two times with rat erythrocytes. The rabbit ALS was titered by adding equal volumes of a thymocyte cell suspension (about 4×10^6 cells/ml) and fresh guinea pig serum to agglutination tubes containing sequential dilutions of rabbit ALS. The cell-serum suspension was incubated for 2hr. at 37°C, and thymocyte viability was ascertained by the trypan blue exclusion test. If the resulting ALS titer (LD_{50}) was less than 1:32, the rabbits were given another injection of thymocytes, rested 4 days and bled. Average titer attained was approximately 1:40 (range 1:32-1:128). The above technique for ALS titration was also employed for titration of the cytotoxic effects of ALS upon isolated Kupffer cells from rat liver.

Normal or control rabbit serum (NRS) was obtained from animals which were injected with Tyrode's solution isovolumetrically. These animals never demonstrated a lymphocytotoxicity titer greater than 1:2. In some experiments, horse anti-rat lymphocyte serum (ALS) and normal horse serum (NHS) were obtained from Microbiological Associates, Inc. (Bethesda) in lyophilized form. The ALS was reconstituted and stored at -20° prior to use. Results obtained with horse ALS were identical to those obtained with rabbit ALS.

In all experiments which evaluate the effects of ALS on the reticuloendothelial (RE) phagocytic function, male Sprague-Dawley rats, weighing between 250 and 300 g, received a single injection of either 1 ml of ALS or 1 ml of NRS prior to evaluation of RES functional activity. The test sera were injected either intravenously or intraperitoneally.

The phagocytic activity of the RES was evaluated by determining the intravascular clearance rates of the gelatinized "RE test lipid emulsion" labeled with I^{131}-triolein, prepared and utilized according to the method of Di Luzio and Riggi (9) and Saba et al. (10). The rats were injected intravenously with the lipid emulsion at a dosage of 50 mg/100 g of body weight. Tail blood samples were obtained every 2 min. for 10 min. and intravascular clearance rates were determined. Liver, lung and spleen samples, obtained 15 min. post-injection of the lipid emulsion, and blood samples were assessed for radioactivity with a Nuclear Chicago Autogamma scintillation system. Results are expressed as percentage of the injected ^{131}I-labeled gelatinized RE test emulsion per g of organ weight (%ID/g) or percentage of injected dose per total organ (%ID/TO).

The *in vitro* techniques employed for assay of liver macrophage phagocytic activity and the evaluation of serum opsonic activity have been previously described (10,11). Briefly, in all *in vitro* experiments 100 units of heparin, 2 mg of gelatinized RE test lipid emulsion, and liver slices weighing approximately 250mg were added to flasks containing incubation media which consisted of either 3 ml of serum or Krebs-Ringer-phosphate buffer (pH 7.4). All flasks were gassed with 95% oxygen and 5% carbon dioxide, then incubated for 30 min. at 37° on a Dubnoff metabolic shaker. The amount of lipid emulsion phagocytized by the Kupffer cells of the liver slices was determined with a Nuclear Chicago Auto-Gamma scintillation system. Results are expressed as the percentage of added ^{131}I-gelatinized RE test lipid emulsion phagocytized per 100 mg of liver slices (%ID/100 mg).

For the studies of the effect of macrophage or lymphocyte transplantation on ALS-induced immunosuppression, rats received 5 daily intravenous injections of either 1 ml of ALS or NRS. On day 5, all rats received, intraperitoneally, 2×10^8 sheep erythrocytes (SRBC). In addition, on day 5, control rats received 1 ml of saline while experimental animals received an intraperitoneal injection of either 2×10^7 macrophages or 2×10^7 lymphocytes in 1 ml of saline. The rats were bled seven days following antigenic challenge. Serum was collected, heat-inactivated for 30 min. at 56°, and titered for hemolysin activity. Serial 2-fold dilutions of the test serum were done using modified

barbital buffer (pH 7.4) as diluent. Lyophilized guinea pig serum (Colorado Serum Co., Denver) reconstituted with 6% sodium acetate in 2% aqueous boric acid solution, was used as a complement source. The tubes were incubated for 30 min. at 37°, then centrifuged. Optical densities were determined, and the 50% hemolysin titer calculated.

Rat peritoneal macrophages were collected four days following an intraperitoneal injection of 10 ml of a 12% sodium caseinate solution. Splenic macrophages were harvested by the adsorption method described by Mosier (12). Kupffer cells were isolated by the method of Pisano et al. (13). The peritoneal and splenic macrophage populations had an average of 10% and 20% lymphocyte contamination, respectively. Histological examination of the isolated Kupffer cells demonstrated a 20%-30% contamination with lymphocyte-like cells.

Lymphocytes were isolated from dispersed splenic cells by allowing the macrophages to adsorb on to Petri plates as described by Mosier (12). Thymocytes were isolated by forcing minced thymus tissue through a 10xx silk screen. Qualitative histological examination of the lymphocyte cellular preparations revealed that a slight (10%) macrophage contamination occurred.

RESULTS

A profound depression in phagocytic function was manifested 4- and 24-hrs. following the intraperitoneal administration of ALS (Table 1). The vascular clearance of gelatinized RE test lipid emulsion was impaired 63% as compared to NRS-treated controls. Hepatic localization of the lipid emulsion had decreased 44 and 33% at 4- and 24-hrs. post-ALS administration, respectively. No significant alteration of pulmonary or splenic phagocytic activity was noted in rats treated with ALS.

In order to delineate the site of ALS-induced RES depression, cellular and humoral components of the phagocytic event were determined. Kupffer cell phagocytic activity and serum opsonic activity were evaluated 4 hrs. after the intraperitoneal administration of ALS (Table 2). In the presence of buffer, Kupffer cells of normal rat liver slices manifested minimal phagocytosis. The presence of normal rat serum induced a 10-fold increase in Kupffer cell phagocytosis as compared to buffer controls. The employment of serum obtained from ALS-treated rats resulted in a degree of phagocytosis that was comparable to that seen with normal rat serum. These experiments demonstrate the presence of normal opsonic activity in ALS-treated rats.

Table 1: Depressant effect of intraperitoneally injected rabbit anti-rat lymphocytic serum on the intravascular clearance and tissue distribution of gelatinized "RE test lipid emulsion".[a]

Treatment	Time[b] (hrs.)	Intravascular t/2 (min)	ORGAN UPTAKE					
			Liver		Lung		Spleen	
			%ID/g	%ID/TO	%ID/g	%ID/TO	%ID/g	%ID/TO
NRS[c]	4	19.4	3.6	48.4	0.8	1.3	5.3	4.5
ALS[d]	4	31.2	2.4	26.7	0.7	1.0	4.8	3.6
ALS[d]	24	31.0	2.6	32.0	0.5	0.8	5.6	4.2

[a]Mean values are derived from 6-8 animals per group.

[b]Hours post-injection of 1 ml test serum.

[c]NRS = Normal Rabbit Serum.

[d]ALS = Rabiit Anti-Rat Lymphocyte Serum.

(s) = significant (<0.05) as compared to NRS injected controls.

Table 2: Effect of intraperitoneally injected rabbit anti-rat lymphocytic serum on opsonic activity as evaluated by *in vitro* hepatic phagocytosis.

Incubation Medium	No. of Experiments	Phagocytic Uptake %ID/100 mg[a]
Krebs-Ringer Buffer	8	2.0 ± 0.8
Normal Rat Serum	10	21.1 ± 2.4
Rat-NRS[b]	10	21.8 ± 3.6
Rat-ALS[c]	10	20.8 ± 4.4

[a]Values expressed as mean ± standard error. Liver slices employed in the *in vitro* humoral recognition factor assay procedure were obtained from normal rats.

[b]Serum obtained from rats which were injected intraperitoneally with normal rabbit serum.

[c]Serum obtained from rats which were injected intraperitoneally with anti-rat lymphocyte serum.

Kupffer cells of liver slices obtained from ALS-treated animals demonstrated a significant impairment in phagocytic activity (Table 3). In contrast to the 21% uptake of the lipid emulsion by liver slices derived from normal or NRS-treated rats, liver slices obtained from ALS-treated rats manifested a 43% impairment of phagocytosis as compared to the NRS-treated liver slices. These findings indicate that the depressive effects of ALS-administration on phagocytosis were dependent upon an ALS-induced alteration of macrophage activity and not upon any humoral or opsonic factor involved in phagocytosis.

Table 3: Inhibition of phagocytic activity of rat liver slices by the administration of rabbit anti-rat lymphocytic serum.[a]

Liver Donor	No. of Slices	Phagocytic Uptake %ID/100 mg[b]
Normal Rat	8	21.3 ± 2.1
NRS-Rat[c]	10	21.0 ± 2.9
ALS-Rat[d]	18	12.1 ± 1.4(s)

[a]Rats were injected with 1.0 ml of test serum 4 hours prior sacrifice and liver extirpation.

[b]Values expressed as mean ± standard error.

[c]Rats injected with normal rabbit serum (NRS).

[d]Rats injected with rabbit anti-rat lymphocyte serum (ALS).

(s) = significant ($P<0.05$) as compared to either normal or NRS-treated rat controls.

Since ALS was shown to alter hepatic macrophage activity, the cytotoxicity of ALS towards Kupffer cells was quantitated (Fig. 1). While NRS was not cytotoxic to isolated Kupffer cells, ALS had a definite killing effect upon these macrophages. The LD_{50} for Kupffer cells, which was 1:16, was significantly less than the LD_{50} of the same preparation of ALS for thymocytes (1:40). However, ALS clearly exerted a cytotoxic effect upon Kupffer cells.

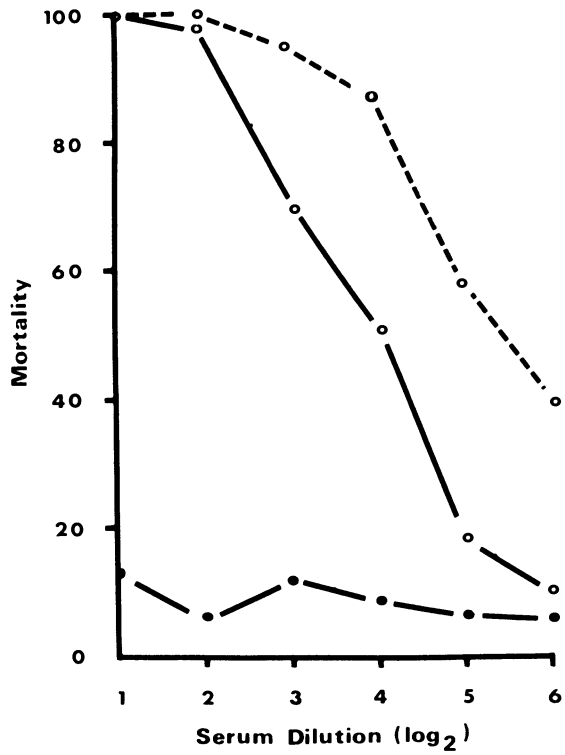

Fig. 1 Comparative cytotoxicity of ALS towards isolated rat Kupffer cells and thymocytes. Mortality was assessed via the trypan blue dye exclusion test. The appropriate cells were incubated for 2 hours in either ALS or normal horse serum and complement. ●-●=Kupffer cells incubated with normal horse serum. (Thymocytes incubated in normal horse serum gave equivalent results.) 0-0=Kupffer cells incubated in ALS; 0--0=thymocytes incubated in ALS.

In view of the participation of the macrophage in immune events and the cytotoxicity of ALS towards Kupffer cells, it was postulated that ALS-induced immunosuppression was mediated by a macrophage dysfunction. In an effort to establish the validity of this concept, the effect of macrophage transplantation on ALS-induced immunosuppression was determined (Fig. 2). The intraperitoneal injection of sheep red blood cells (SRBC) into untreated control rats resulted in an hemolysin titer of 1:67. The administration of NHS prior to antigenic challange was associated with a 37% reduction in antibody titer while ALS produced a 90% reduction in the humoral immune response of the rat.

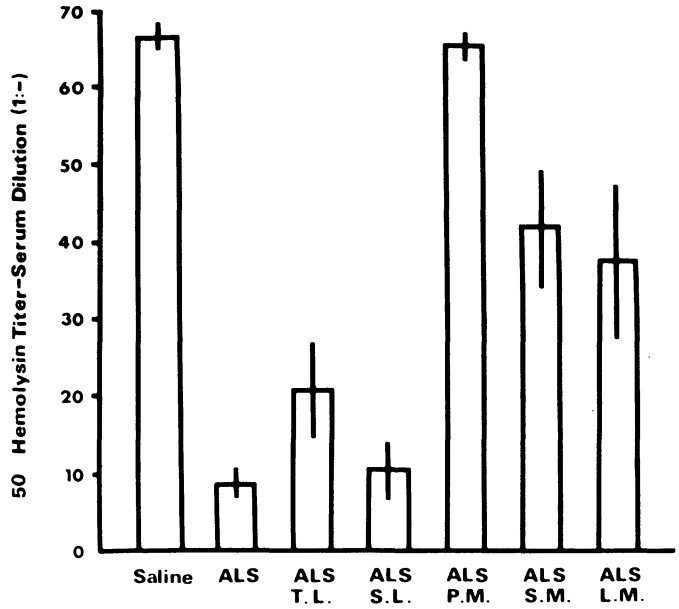

Fig. 2 Reversal of ALS immunosuppression by the transplantation of various macrophage populations. Rats received 5 daily injections of ALS, on day 5 all animals received 2 x 10^8 sheep erythrocytes. In addition, on day 5, control rats received 1 ml of saline, while experimental animals received either 2 x 10^7 macrophages or 2 x 10^7 lymphocytes. The 50% hemolysin titers were determined 7 days following challenge with sheep erythrocytes and are expressed as the mean ± standard error of 8 experiments. T.L.=thymic lymphocytes; S.L.=splenic lymphocytes; P.M.=peritoneal macrophages; S.M.=splenic macrophages; L.M.=liver macrophages.

Transplantation of either thymocytes, splenic macrophages or lymphocytes, peritoneal macrophages, or Kupffer cells did not affect the hemolysin titer produced by the injection of SRBC into control rats. The transplantation of these diverse isolated cell populations did not induce an hemolysin response in rats which did not receive SRBC.

The transplantation of the various macrophage populations into ALS-treated animals resulted in either a complete or partial reversal of ALS-induced immunosuppression (Fig. 2). Peritoneal

macrophages were capable of completely reversing the immunosuppressive effects of ALS on hemolysin formation. Splenic macrophages were capable of inducing a 67% restoration in antibody production while Kupffer cell transplantation resulted in a comparable 58% restoration of immune activity in ALS-treated animals. In contrast to the ability of macrophages to reverse ALS-immunosuppression, transplantation of either thymocytes or splenic lymphocytes did not significantly alter the ALS-induced immunosuppressed state (Fig. 2).

DISCUSSION

The intraperitoneal injection of ALS resulted in a profound depression of the RES as manifested by the prolongation of the intravascular clearance of gelatinized RE test lipid emulsion. The depressed clearance rate was associated with a selective impairment of hepatic localization of the emulsion. The in vitro liver slice studies established that the depression of RES function was not mediated by an alteration of serum humoral factor or opsonic activity but was directly associated with a cellular impairment. The ALS-induced macrophage impairment was further demonstrated by the rather profound cytotoxicity of ALS to Kupffer cells.

The phagocytic depression induced by ALS is of a similar degree observed with either methyl palmitate (14) or x-irradiation (15). However, in contrast to the absence of an impairment in splenic phagocytosis observed with ALS-treated animals, both methyl palmitate and x-irradiation induce an impairment of splenic localization of foreign colloidal materials (14,15). Since ALS, methyl palmitate and x-irradiation are all immunosuppressives, it is possible that macrophage impairment participates in the immunosuppression induced by these agents. Thus, Feldman and Gallily (16) have proposed that x-irradiation-induced immunosuppression is mediated by a loss of macrophage function. Wooles and Di Luzio (14) implicated the macrophage as the mediator of methyl palmitate immunosuppressive activity. The present observation that ALS possesses a cytotoxicity towards isolated Kupffer cells suggested that ALS-induced immunosuppression may also be, in part, macrophage mediated. Since isolated peritoneal, splenic and hepatic macrophages were capable of either totally, or partially, reversing the immune depression induced by ALS administration, it would appear that macrophage cytotoxicity is, indeed, an important factor in ALS-immunosuppression. Since all three macrophage populations contained some degree of lymphocyte contamination, one might predicate that the presence of lymphocytes contributed to the restoration in immune activity. However, since isolated splenic or thymic lymphocytes did not

possess the capability to promote immunogenesis in ALS-treated animals, it can be proposed that the ALS-induced impairment of macrophage function is an important facet of ALS-induced immunosuppression.

Since patients receiving immunosuppressive therapy are more susceptible to infection and neoplastic growth (2-4), it is entirely possible that the anti-macrophage activity of certain therapeutic agents, such as ALS, may be the detrimental factor in the alteration of host defense. If it is conclusively shown that ALS is immunosuppressive because of its anti-macrophage activity, it may be necessary to develop agents which are devoid of anti-macrophage activity. Such agents may block transplant rejection mechanisms, while allowing other host-defense systems to remain intact, thus maintaining homeostatic mechanisms against infections and neoplasia.

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DIFFERENTIAL RESPONSES OF MACROPHAGE PRECURSORS AND ANTIBODY FORMING CELLS TO IMMUNIZATION

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INTRODUCTION

Participation of macrophages in immune responses has been shown in many experiments using various methods (1)(2)(3)(4)(5). It has also been demonstrated that antibody forming cells and their precursors proliferate as a result of injection of antigen (6)(7). As for macrophages and their precursors, this fact has not yet been fully elucidated. The technique of cloning spleen, bone marrow and embryonic liver cells in soft agar (8)(9)(10) now enables quantitative studies of macrophages as well. In this technique certain cells from these hematopoietic organs can multiply in a soft agar medium to which conditioned medium is added (11). This leads to formation of macroscopic cell colonies of four types: macrophages, granulocytes, blast cells, and a mixture of macrophages and granulocytes (9)(12). It has been shown that macrophage colonies originate from a single precursor cell (11). Therefore each macrophage colony in the soft agar represents a precursor cell.

Our experiments were undertaken to study whether immunization of mice with foreign red blood cells is followed by quantitative changes in the macrophage precursor cells (MPC) in the spleen and whether these cells are controled by the same mechanism which controls hemolytic plaque forming cells (PFC) and their precursors.

MATERIALS AND METHODS

The same basic technique was used in all the experiments.

Male SWR mice aged 8–12 weeks were immunized with sheep or horse red blood cells (RBC) suspended in a volume of 0.5 ml saline. At different intervals after immunization the spleens were removed and converted into suspensions of single cells. Live nucleated cells were cloned in soft agar (11) and assayed for hemolytic PFC (13).

RESULTS

A. Response of Splenic MPC to immunization with 10% Sheep Red Blood Cells (S-RBC).

S-RBC in 10% suspension were injected intraperitoneally

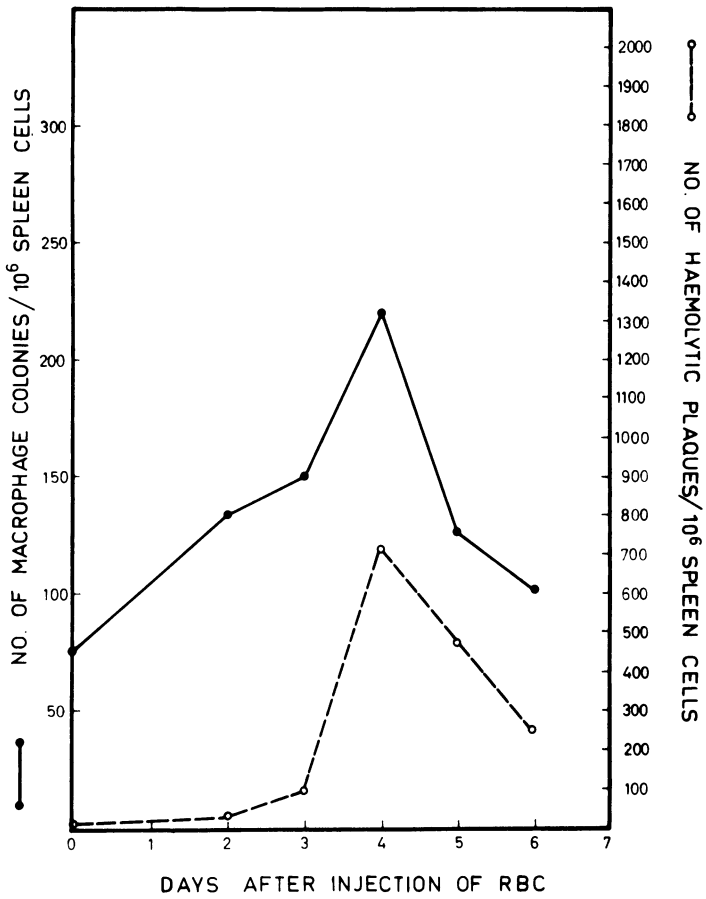

Fig. 1. Quantitative changes of macrophage colonies and PFC after immunization with 10% S-RBC.

and spleens removed at 24 hour intervals were treated as mentioned above. The results of such an experiment can be seen in Fig. 1. As shown in this fig. the number of macrophage colonies gradually increased until day 4 after immunization. This was followed by a decrease in their number. A rapid increase was also seen in the number of hemolytic PFC which also reached a peak at day 4 after immunization. No such clearcut increase was observed in the granulocyte and blast cell colonies.

B. Relationship between Concentration of S-RBC and Response of MPC.

In order to examine whether a correlation exists between the amount of antigen injected and the increase in the number

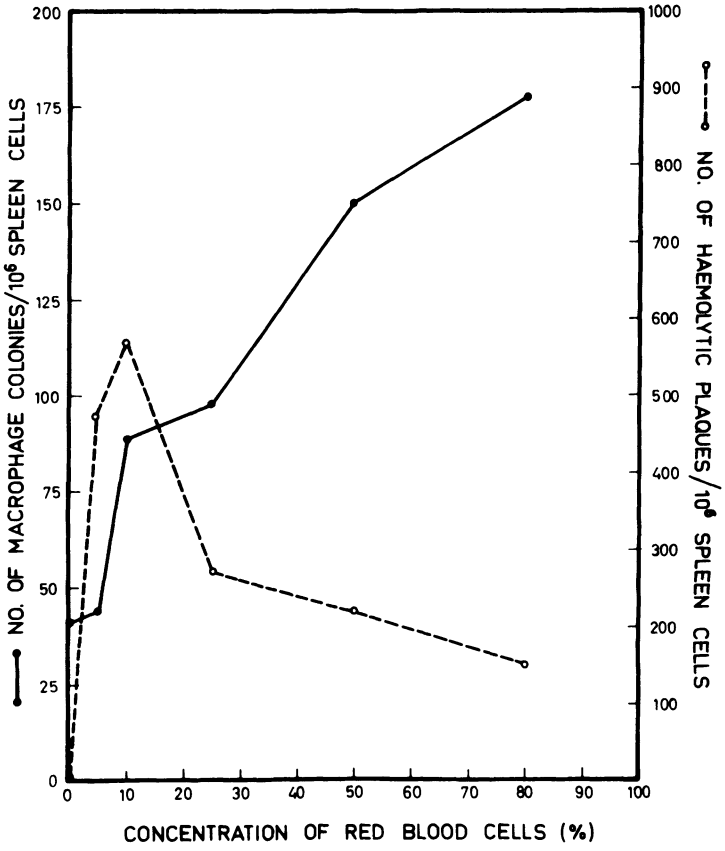

Fig. 2. Response of macrophage colonies and PFC to immunization with increasing concentrations of S-RBC.

of MPC, increasing amounts of S-RBC from 5% to 80% were used for immunization. Four days later the spleen cells were cloned in soft agar and assayed for PFC. The results obtained (Fig. 2) indicate that an increase in the concentration of S-RBC starting from 10% causes an increment of number of MPC. Concerning the PFC, the lower concentrations of S-RBC, 5%-10%, elicited the highest response in their number whereas any excess over 10% caused a decrease.

C. Reaction of MPC and Antibody Forming Cells to Immunization with S-RBC in Secondary Response and in Tolerant Mice.

The difference in response of both types of cells to immunization with S-RBC may suggest that different mechanisms control these two types of cells. In order to elucidate further this problem two groups of experiments were designed.

Table 1

Cellular and Humoral Reaction to Immunization with S-RBC in Primary and Secondary Responses

Type of reaction	Days after immunization				
	0	1	2	4	6
Primary response:					
No. of macrophage colonies per 10^6 spleen cells	91	NT	NT	192	NT
No. of PFC per 10^6 spleen cells	2	NT	NT	850	NT
Titer of hemagglutinins (Reciprocal)	0	NT	NT	32	NT
Secondary response (60 days):					
No. of macrophage colonies per 10^6 spleen cells	188	193	191	200	178
No. of PFC per 10^6 spleen cells	5	4	45	310	43
Titer of hemagglutinins (Reciprocal)	256	256	512	4096	8192

In the first group of experiments the quantitative changes of MPC in the secondary response were tested. The results of such an experiment (Table 1) show that PFC and hemagglutinating antibodies behave as expected, namely by increasing their numbers and titer whereas MPC showed only a very moderate increase.

In the second group of experiments mice were made tolerant to S-RBC by means of cyclophosphamide (14). Results of such treatment (Table 2) caused a specific reduction in hemolytic PFC for S-RBC, but had no effect on MPC. Significantly also, mice tolerant to S-RBC showed the normal increase in PFC for horse RBC (H-RBC), after immunization with this antigen.

Table 2

Effect of Cyclophosphamide on Macrophage Colonies and PFC

Treatment*	No. of macrophage colonies per 10^6 spleen cells	No. of PFC per 10^6 spleen cells	
		Sheep	Horse
Cyclophosphamide**:			
Control	127	2	2
S-RBC	297	523	2
H-RBC	293	3	254
Sheep RBC:			
Control	83	7	1
S-RBC	174	358	2
H-RBC	272	5	330
Cyclophosphamide** + S-RBC:			
Control	81	7	9
S-RBC	353	83	3
H-RBC	570	11	350
No treatment:			
Control	90	1	2
S-RBC	243	542	2
H-RBC	259	1	227

* Treatment of mice 21 days before second immunization with 50% RBC. Mice sacrificed 4 days after second immunization.

** 3 mg per mouse.

D. Effect of a Mitotic Inhibitor on Number of MPC

The data presented confirm the suggestion that MPC are controled by different mechanisms than hemolytic antibody forming cells. As for the latter type of cells it was shown that immunization of mice with S-RBC caused proliferation of PFC and their precursors (6)(7). It was of interest to find out whether the increase in MPC is also due to proliferation. To answer this question, the mitotic inhibitor vinblastine (7) was injected into mice 72 hours after immunization with S-RBC. Twenty-four hours later the spleen cells were cloned in soft agar and assayed for PFC (Table 3).

From the results obtained it can be seen that both types of cells, MPC and PFC responded with a decrease in their numbers, thus suggesting that the increase in MPC after immunization with S-RBC reflects cell proliferation as in the case with PFC.

DISCUSSION

It has previously been shown that colonies grown in soft agar are predominantly macrophage colonies (9)(12). It has also been shown that each macrophage colony originates from a single precursor cell which proliferates and maturates in vitro (15). The experiments described above have demonstrated that immunization of mice with foreign RBC is followed by an increase in number of MPC in the spleens as judged by the number of macrophage colonies grown in soft agar. Quantitative changes also occurred in the antibody forming cells. Both types of

Table 3

Effect of Vinblastine on Macrophage Colonies and PFC

Treatment	No. of macrophage colonies per 10^6 spleen cells	No. of PFC per 10^6 spleen cells
Control	74	1
Control + vinblastine*	88	0
S-RBC + vinblastine*	103	132
S-RBC (day 3)	215	245
S-RBC (day 4)	232	570

* 50 µg per mouse, injected 24 hours before sacrifice.

cells reached peak numbers at day 4 after immunization. The results of experiments with the mitotic inhibitor vinblastine suggested that the quantitative changes of these two types of cells is probably due to cell proliferation. On the other hand, the fact that excess of RBC caused an increase in MPC and a decrease in antibody forming cells suggest that different mechanisms control these two cell populations. Such a possibility was further supported by the different quantitative reaction of MPC and antibody forming cells to immunization with S-RBC in the secondary response. The two types of cells also showed a different response when tolerance for S-RBC was induced by administration of cyclophosphamide together with the antigen. These data support the concept that macrophages and antibody forming cells are controlled by different mechanisms.

Fig. 3 presents a theory intended to explain the mechanism controlling macrophage proliferation. It has been shown that mature macrophages can produce an inhibitor which prevents multiplication and maturation of MPC to macrophages (15). The foreign RBC injected into mice are presumably ingested by mature macrophages. Such cells may be considered to be "blocked" and they do not produce the inhibitor or produce it in insufficient amounts thus permitting proliferation of macrophage precursors to mature "unblocked" cells which can release the inhibitor to prevent any further proliferation of precursor cells.

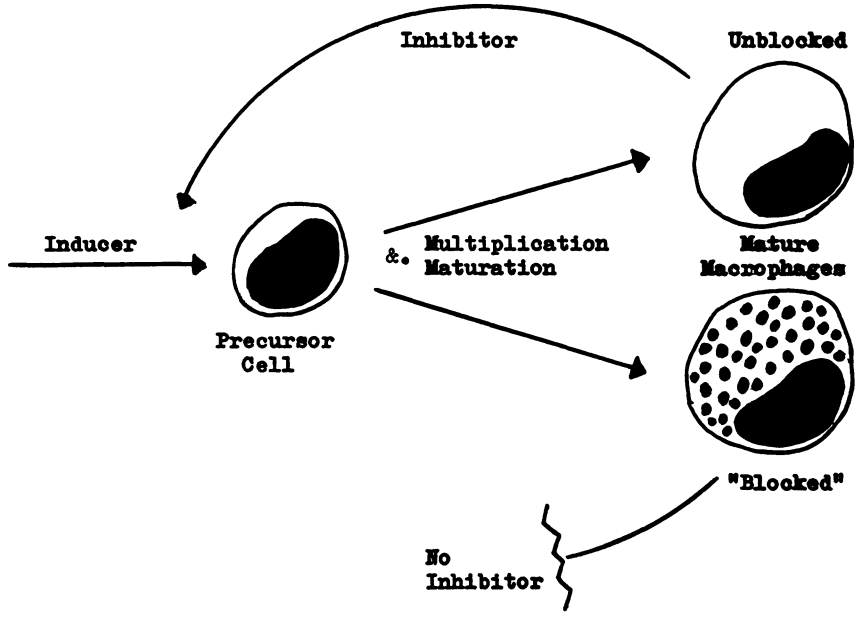

Fig. 3. Theory explaining macrophage proliferation as result of immunization with S-RBC.

SUMMARY

A quantitative assay of MPC, based on cloning splenic cells in soft agar, was used for evaluating the involvement of macrophages in immunological processes. Changes in numbers of splenic MPC resulting from intraperitoneal injection into mice of S-RBC, in parallel with assay of splenic PFC were determined. Immunization with 10% S-RBC increased splenic MPC with the peak being reached 4 days after injection. When the quantity of RBC injected was increased, there was a further rise in MPC. By contrast, the number of splenic PFC decreased when the amount of S-RBC exceeded 10% per mouse. In secondary responses to S-RBC. a rise in PFC and in hemagglutinins occurred as expected. On the other hand, only a moderate increase in MPC was associated with the secondary response. In mice made "tolerant" to S-RBC by means of cyclophosphamide numbers of PFC were low, whereas MPC increased similarly as in controls. Finally, injection of the mitotic inhibitor vinblastine 72 hours after immunization with S-RBC decreased the numbers of PFC as well as MPC. This last observation suggests that the increase in numbers of MPC reflects proliferation of cells, similarly as in the case with PFC. On the other hand, all other data support the concept that macrophages and antibody forming cells are controlled by different mechanisms.

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LIGHT AND ELECTRON MICROSCOPIC CHANGES IN LYMPH NODES AFTER LOCAL APPLICATION OF HETEROLOGOUS ANTITHYMOCYTE AND ANTI-MACROPHAGE SERUM

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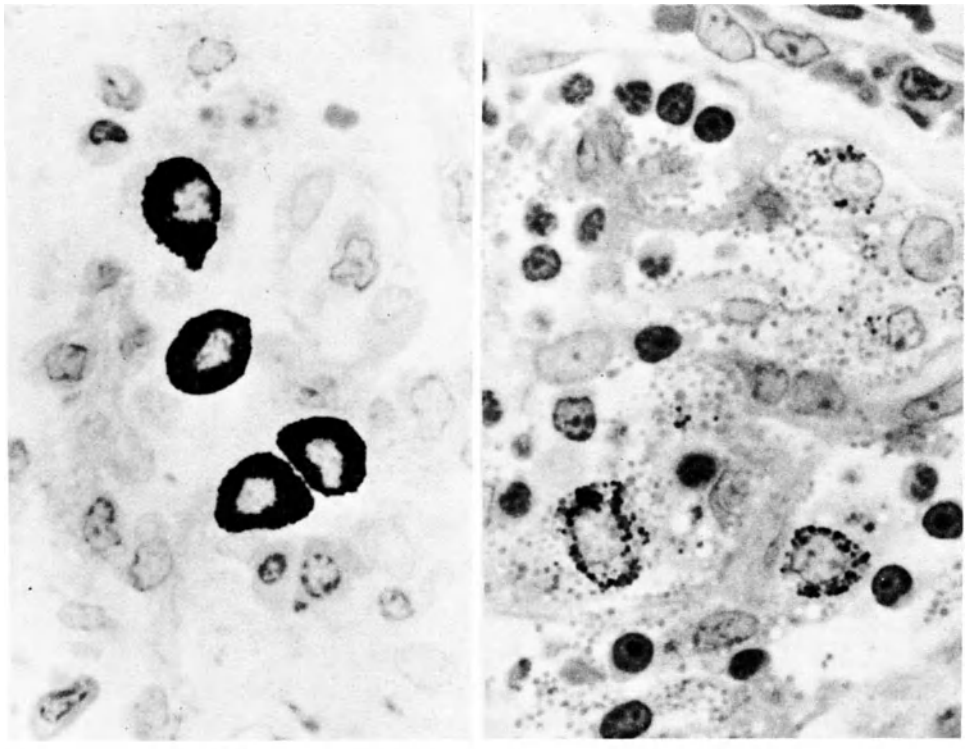
I. Introduction

Two morphologically different cell types are involved in the production of immunological sensitization against many antigens, namely, macrophages and lymphocytes (e.g., 4, 15, 22, 3). Applying antisera specifically directed against either of these cells may result in a delay or suppression of the primary immunological response. This has been proved to be true for antithymocyte sera (ATS) (e.g., 23, 8, 14, 1). Also, studies on the *in vivo* action of antimacrophage sera (AMS) revealed prolongation of allogeneic skin graft survival time in rats (17). The present work intends to elucidate morphologically whether or not the apparently similar effect of these antisera (prolongation of skin graft survival time) is caused by a specific action on different target cells (lymphocytes and macrophages) *in vivo*. Therefore, we studied the morphological alterations in popliteal lymph nodes following local application of either antiserum. The electron microscopic observations presented here are added to recently published light microscopic and enzyme histochemical studies (19, 20).

II. Material and Methods

Rabbits were injected i.v. for 3 weeks with 6×10^7 washed rat peritoneal macrophages harvested one day after i.p. injection of 10 ml paraffin oil, or with 6×10^9 thymocytes of excanguinated rats. Seven days after the last injection antisera were prepared. Each antiserum was absorbed exhaustively with rat RBC. In addition, ATS was absorbed with peritoneal macrophages and AMS with thymocytes until no cytotoxic activity against the cell type used for absorption could be detected.

About 4-month-old male LEW and CDF inbred rats were injected s.c. into the one hindpad with ATS (0.08 ml per 100 gm/bw thymocyte cytotoxicity titer 1:180) or AMS (0.16 ml per 100 gm/bw, macrophage cytotoxicity titer 1:90). As control rabbit normal serum (NRS) in corresponding dosage was given into the other pad. Popliteal lymph nodes were then removed after different time intervals (5 min, 10 min, 20 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr, 24 hr, 2 days, 4 days, 5 days, 6 days, 7 days, 8 days). After fixation with a mixture of glutaraldehyde and paraformaldehyde (10) and postosmification with 1% veronal-acetate buffered OsO_4 (pH 7.2) the samples were embedded as usual for electron microscopy in araldite. Sections for light microscopy were stained with azur-A-methylene blue. Thin sections for electron microscopy were cut on a Porter-Blum Ultramicrotome with glass knives. Thin sections were stained for electron microscopy with uranyl acetate and lead citrate and evaluated at a Siemens Elmiscop I with primary magnifications between 3000 and 25,000 x.



(a) (b)

Fig. 1. (a) Tissue mast cells in lymph node sinus 1 hr after injection of ATS. (b) Degranulation of tissue mast cells 5 min after AMS Methylene blue Azur A (1200 x).

III. Results

Following a single injection of either antiserum, specific alterations could be observed within the first 4 hr, whereas NRS-treated lymph nodes revealed no noticeable structural changes at this time.

Following injection of ATS, light microscopically the most impressive changes were observed after 2 hr: severe lymphocyte depletion of the lymph node cortex, especially below the marginal sinus and in the paracortical zone near the lymph node medula. Besides, macrophages are observed in the medullar sinuses having phagocytosed large amounts of pycnotic cells. Macrophages appeared undamaged as previously shown by acid phosphatase reaction (20).

Following injection of AMS, all tissue mast cells of the lymph node medulla showed complete degranulation within 5 min (Fig. 1). This was followed by a massive edema of the sinuses accompanied by fibrin exudation. Sinus macrophages showed marked cytological alterations 1-2 hr after injection: hydropic swelling of cytoplasm,

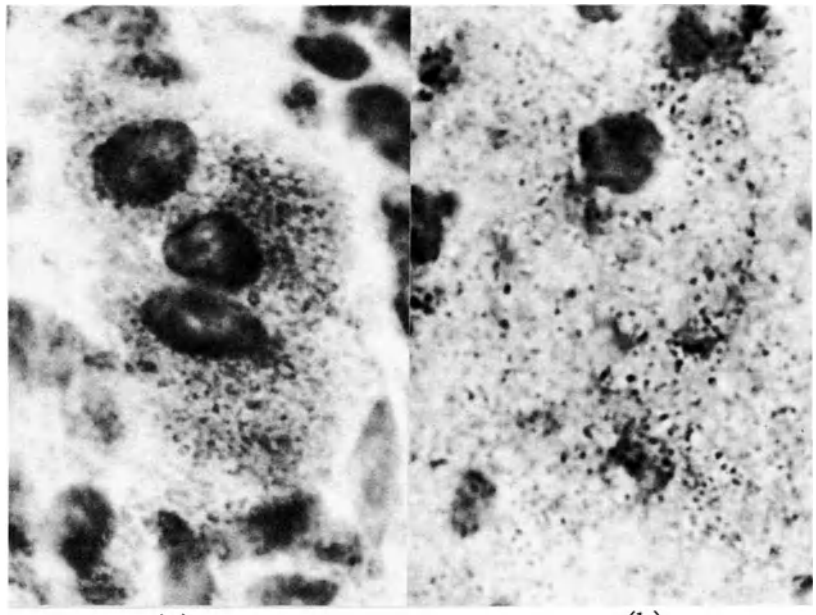


Fig. 2. (a) Acid phosphatase reaction 2 hr after injection of NRS. Sinus macrophages. (b) Acid phosphatase reaction 2 hr after injection of AMS. Diffuse enzyme distribution within the sinuses (1200 x).

appearance of intracytoplasmic vacuoles, and a gradual disappearance of the cell limits. In many instances nuclei of the sinus macrophages were pycnotic or showed signs of karyolysis. Acid phosphatase reaction revealed disappearance of enzyme positive granules from the cytoplasm with appearance of diffuse enzyme distribution in the sinus lumen. These alterations were found mainly in the marginal and medullar sinuses near the lymph node hilus (Fig. 2).

Following the application of ATS, alterations of lymph node structure were found electron microscopically within 10 min but were more pronounced after 20-30 min. Macrophages of the medullar pulp cords and sinuses were incorporating or had phagocytosed lymphocytes with intact morphological structures. These lympho-

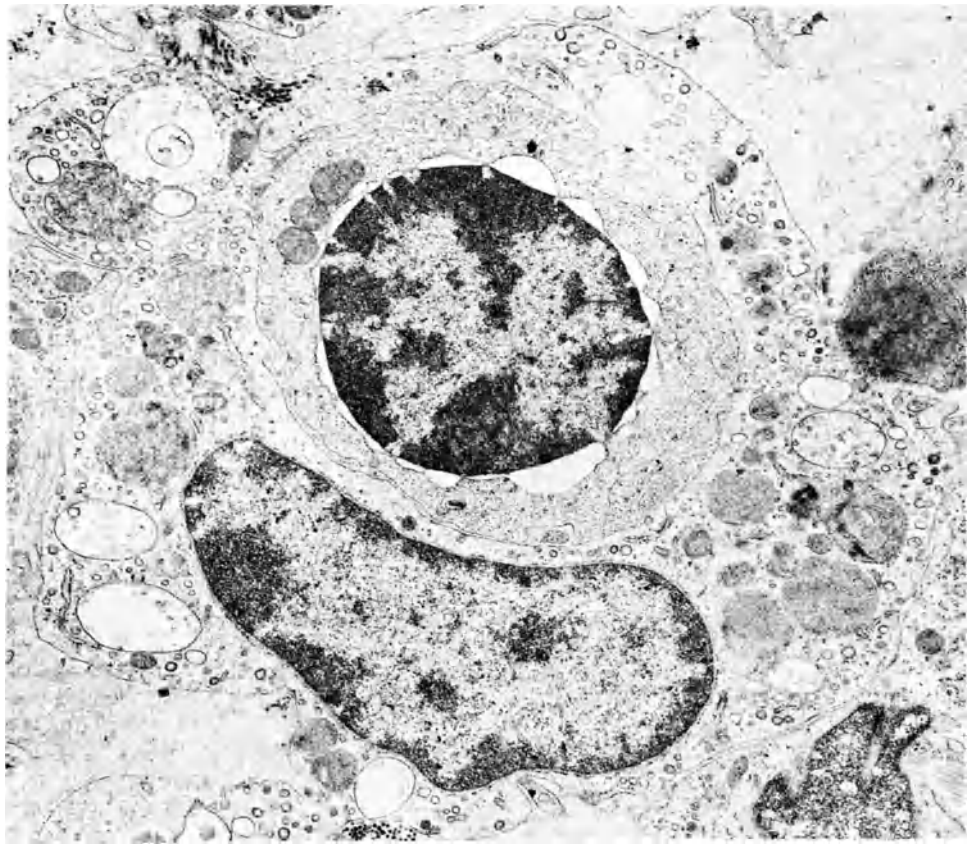


Fig. 3. Sinus macrophage ingesting a lymphocyte showing fractionation of the cytomembrane and distention of the perinulcear cleft 30 min after ATS treatment (12,000 x).

cytes revealed undamaged cytoplasmic membranes and the typical electron dense chromatin pattern of the cell nuclei. Frequently, fine granular electron dense material became visible between the members of the phagolysosome and the surface membrane of the phagocytosed lymphocyte (Fig. 3). Increasing with time, the phagocytosed cells showed signs of cellular degeneration such as distention of the perinuclear cleft, fragmentation of the cytoplasmic membranes, and sequestration of cytoplasmic components. With time the number of phagocytosed cells increased rapidly. One or two hours after ATS nearly all sinus macrophages had incorporated at least one lymphocyte. Phagocytosed lymphocytes revealed densification of

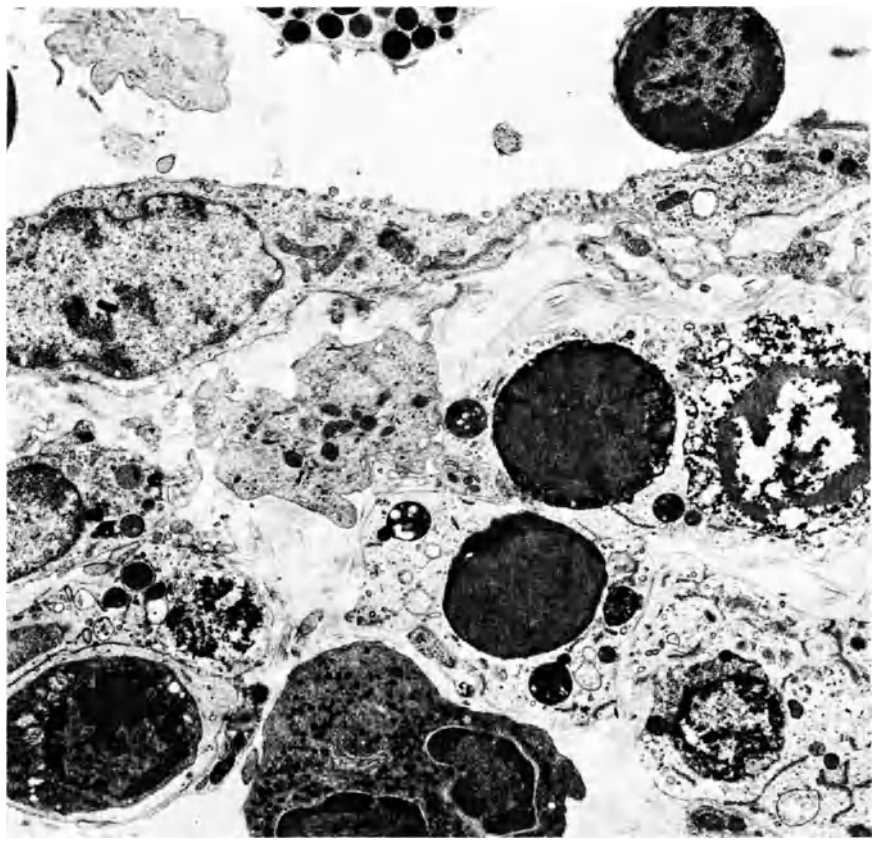


Fig. 4. Pump cord (bottom) and sinus (top) of a lymph node 1 hr after ATS treatment. Note pycnotic and cytolytic lymphocytes within the cytoplasm of pulp cord macrophages. Within the sinus lumen there appear pycnotic lymphocyte nuclei without cytoplasm (7500 x).

nucleus and cytoplasm or complete disintegration of the cell. Such macrophages were detectable in the pulp cords (Fig. 4). However, cells with higher phagocytic activity were found in the sinus lumina. Occasionally, macrophages were observed just passing through the sinus walls. Later, i.e., 4 hr after the injection of ATS, most of the sinus macrophages contained only dense bodies occasionally still containing rests of cytoplasmic structures.

Besides free lymphocytolysis with disintegration of cellular membranes, pycnosis or karyorrhexis were visible within sinus and the lymph node cortex.

After application of AMS, the following electron microscopic alterations of mast cells were visible after 5 min. Granules showed disintegration with loss of electron density and fusion of their membranes, while their content was liberated into the sinus lumina. During this degranulation, mast cells may lose their cytoplasm. At this early time the neighboring sinus macrophages appeared to be morphologically intact and phagocytosed the liberated mast cell granules. Mast cell degranulation was followed by a massive edema and fibrin deposition within the sinus and lining the cytoplasmic processus of the reticulum cells of the lymph node cortex.

After 20-30 min the first alterations of macrophage cytoplasm became detectable: severe intracytoplasmic edema developed without altering the chromatin structure of the nucleus. Electron density of the cytoplasm was strongly reduced. The cytoplasmic membranes of damaged cells showed disintegration and fragmentation of the cytomembranes (Fig. 5). Later, 1-2 hr p.i., a complete cytolysis of macrophages with appearance of cellular debris within the sinus lumina became detectable. Cortical reticulum cells, especially those localized in the apracortical zone showed a marked intracytoplasmic edema and only occasional cytolysis.

Rarely, however, were macrophages phagocytosed by other macrophages which may show signs of cytoplasmic degeneration.

More than 4 hr after injection of ATS or AMS no additional cell damage was observed. An explosive inflammatory reaction became noticeable, infiltration of the whole lymph node with granulocytes and monocytes removing the cellular debris. After 48 hr the lymph node structure was repaired. From that time on the typical lymph node reaction to foreign protein with plasmocytosis in the pulp cords prevailed. At any rate, no significant morphological differences between ATS-, and AMS-, and NRS-treated lymph nodes could be recognized.

IV. Discussion

Our findings show that both antisera induce obvious but different morphological alterations in the treated lymph nodes. The application of ATS results in phagocytosis of intact or damaged lymphocytes as well as cytolysis of lymphocytes that have not been phagocytosed. This evokes a pronounced depletion of cortical lymphocytes. Macrophages, however, remain intact. The application of AMS on the other hand, initiates mainly a destruction of macrophages without visible signs of lymphocyte damage.

Three main effects of ATS on the lymphocytes have been discussed, cytolysis (8); opsonization, i.e., activation of lymphocytophagocytosis induced by the reaction of antibodies with lymphocyte surface antigens (11, 12, 13); and blast transformation

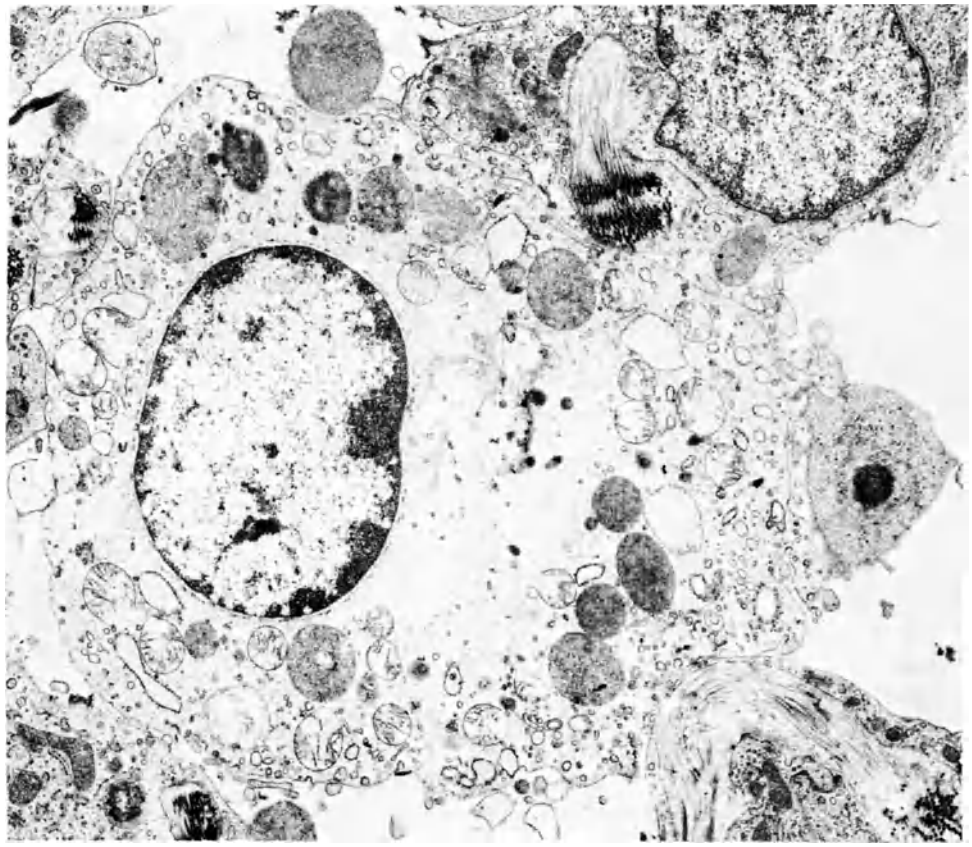


Fig. 5. Early damage of sinus macrophage 30 min after AMS treatment. Cytoplasmic edema and disintegration of the cytomembrane. Nucleus with intact chromatin structure (12,000 x).

(5, 16). In our in vivo experiments the opsonizing action of ATS seems to prevail. However, it must be pointed out that after time intervals of more than 1 hr, it cannot be decided whether or not the phagocytosed cells were still intact before phagocytosis. In this context, the studies of Martin (11, 12, 13) may be cited in which he showed the phagocytosis of ATS-pretreated lymphocytes predominantly by von Kuppfer cells in the liver. The reduction of the phagocytic capacity of the RES following ATS administration may be interpreted along this line of reasoning (6).

With regard to the depletion of lymphocytes, mainly of the paracortical zone of the lymph node, it may be mentioned that similar observations have been made by Taub and Lance (18), Fioredonati et al. (2), and Tridente and v. Bekkum (21).

AMS administration in vitro has been shown to induce inhibition of phagocytosis and decrease of macrophage adhesiveness to surfaces (22, 7, 9). Sonntac and Muller-Ruchholtz (17) recorded a high cytotoxic titer of AMS on macrophages in vitro and prolongation of skin allograft survival time in rats. Thiede et al. (19, 20) described morphologic changes after in vivo application of AMS. Our results show that macorphages and reticulum cells of the lymph node appear to be the main target cells of this antiserum. Apparently the inhibitory and destructive action of AMS on macrophages leads to the predominance of free cytolysis of the target cells, though we found occasional phagocytosis of sinus macrophages.

The mast-cell-destroying activity of AMS which preceded the morphologic alteration of macrophages seems likely to be caused by antibodies cross-reacting with mast cell surface antigens. However, the possibility cannot be excluded that the production of antibodies against mast cells has been stimulated by mast cell contamination of the cell suspension used for immunization.

As mentioned, both ATS and AMS have an immunosuppressive effect on allogenic rat skin grafts (17). Although in this experimental model inhibition of immunological sensitization cannot be considered as the only possible explanation of the suppressive effects of ATS and AMS, our results offer the morphological basis for interpreting the quite different actions of these antisera as affecting the afferent limb of an immunological reaction.

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THE MEASUREMENT OF CELL-MEDIATED IMMUNITY IN MAN

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The Aschoff node was described in 1904(1) by the pathologist whose life and work is being commemorated here in Freiburg. This focus of chronic inflammation is probably the end result of a cell-mediated immune response but medical science today is still lacking a rapid and reliable in-vitro method of measuring this most important reaction.

The humoral immune response, which is phylogenetically more recent than that mediated by lymphocytes, is characterized by the production of immunoglobulins. Humoral immunity can therefore be measured accurately and relatively simply since specific proteins are liberated into solution by populations of sensitized cells. In contrast, the degree of sensitization of lymphocyte populations is not measurable by simple titration and thus it is our task to supply the sophistication which in humoral immunity has been provided for us already by Evolution.

Sensitized lymphocytes are known to interact with their corresponding antigen to produce lymphokines. These soluble substances are biologically active and can cause lymphocyte transformation(2), inhibition of migration of macrophages(3), and a variety of other phenomena involving cells and tissues. Measurement of lymphokine activity, therefore, would seem to offer some possibility of determining the degree of sensitization of a population of lymphocytes to an antigen. Assay of the biological activity, however, is the critical stage of such a determination, and I have been investigating the movement of cells from capillaries and the spreading of mouse macrophages on glass to assess their value in the measurement of cell mediated immunity in man.

METHODS

Leucocyte and Macrophage Suspensions

Blood was obtained by venepuncture from a series of healthy young adults and immediately transferred to glass bottles containing heparin to give a final concentration of 5 i.u./ml. The leucocytes were separated by sedimentation with Dextran 250 (Pharmacia, Sweden), washed three times with Eagle's medium and the density of the cell suspension determined by counting.

Random bred Swiss albino mice were used as a source of macrophages. 2ml of Eagle's medium containing 10 per cent of heat-inactivated calf serum and 5 i.u./ml of heparin was injected intraperitoneally immediately after killing an animal by cervical dislocation. The fluid was aspirated and the number of cells in the suspension was determined by counting; when the macrophages were required for migration experiments the cells were washed three times with Eagle's medium containing 10 per cent of heat-inactivated calf serum.

Movement of Leucocytes and Macrophages from Capillary Tubes

75mm glass tubes of 1mm internal diameter were filled by capillarity to a mark 40mm from one end with leucocyte or macrophage suspensions of known cell density. The tubes were plugged with Sealease (Clay Adams, U.S.A) and the suspensions were sedimented by centrifuging at 50 x g for 5 min. The capillaries were then cut at the cell-liquid interface and mounted with Vaseline in Perspex observation chambers(4) so that the cut ends rested on 1/2" diameter glass coverslips which were also attached to the bottom of the cell interior with the same blob of Vaseline. The chambers were closed with glass coverslips, filled with Eagle's medium containing the substance under test and incubated for 16 h at 37°C. The areas occupied by cells that had moved from the capillaries were measured by projection.

Leucocyte-Antigen Cultures

Leucocyte suspensions were mixed with a solution of P.P.D. in Eagle's medium so that the ratio of lymphocytes to antigen was 10^4 cells/µg P.P.D. The P.P.D. was obtained as a powder from the U.K. Ministry of Agriculture's Central Veterinary Laboratory. The suspensions were incubated at 37°C for 16 h after which the supernatants were removed by centrifuging at 50 x g for 5 min. The ability of the culture supernatants to inhibit the spreading

of macrophages on glass was determined and each was tested together with two controls. One control (cell control) consisted of a supernatant from a suspension of the same leucocytes which had been incubated without antigen and this was included because culture supernatants from human polymorphs have been shown to inhibit macrophage migration(5). The second control (antigen control) was a solution of P.P.D. of equivalent concentration that had been incubated in the absence of leucocytes.

The Spreading of Macrophages on Glass

Freshly harvested mouse macrophage suspensions were divided into aliquots and to some of these were added cell and control supernatants. The suspensions were allowed to settle at 37°C on glass coverslips in an atmosphere of 5 per cent CO_2 saturated with water vapour in a water-jacketed modification of the settling chamber described by Brogan(4). After 10 min coverslips were removed and sealed on to the Perspex observation chambers, the interiors of which were filled with Eagle's medium or with the corresponding test or control supernatant. All culture fluids contained 10 per cent of heat-inactivated calf serum. The chambers were inverted so that the cells were not subjected to distraction by gravity and incubated at 37°C for 16 h. The percentages of spreading were measured by counting the proportion of round cells (Fig.1) in the macrophage populations with an inverted phase contrast microscope.

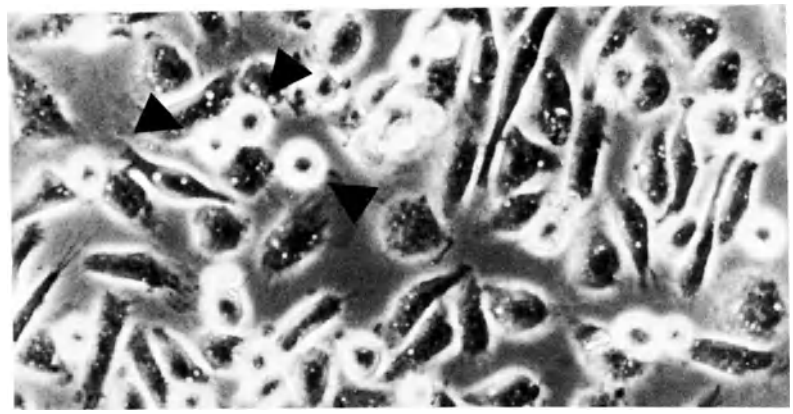

Fig. 1. The spreading of mouse macrophages on glass. The percentage of spreading in a preparation was estimated by counting the proportion of round cells in the population. Examples of round cells are shown on the photograph by arrows and are characterized by their prominent phase haloes. Phase contrast X 250.

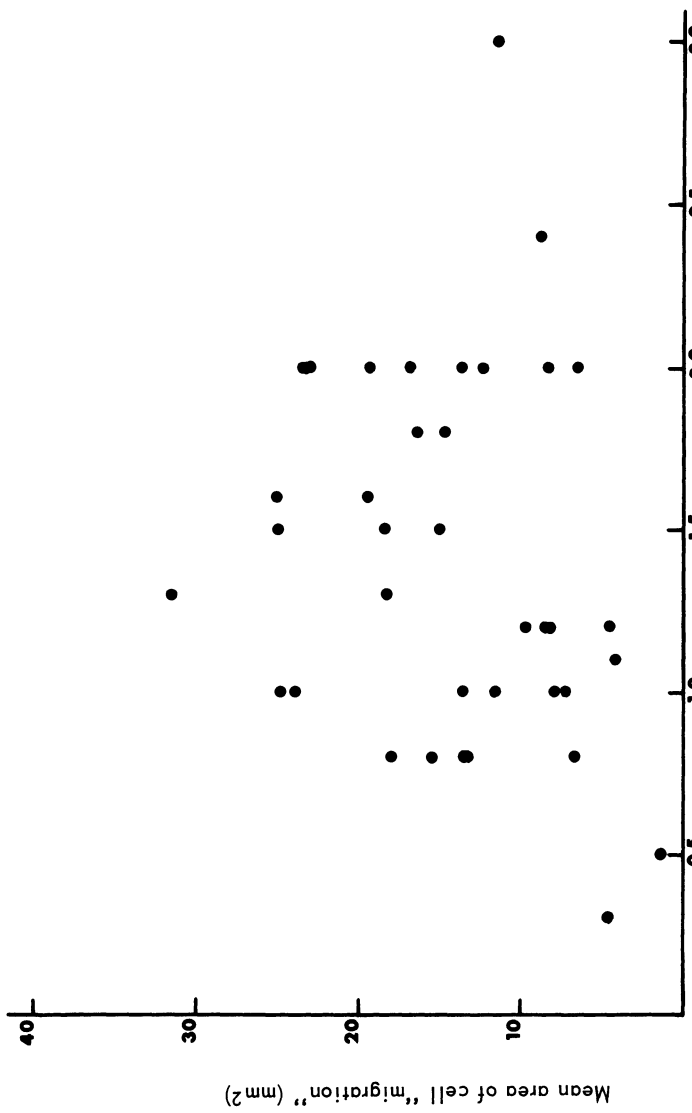

Fig. 2. Variation in the areas of "migration" of peripheral blood leucocytes from capillary tubes after incubation at 37°C for 16 h in serum-free Eagle's medium. The data were obtained from 38 normal human subjects.

RESULTS

Movement of Leucocytes and Macrophages from Capillary Tubes

Considerable variation was encountered in the areas of "migration" of human leucocytes in the capillary tube preparations (Fig.2). The variation was unrelated to the numbers of cells in the capillaries and was unaffected by the presence of serum in the medium or by alterations in pH within physiological limits. The average coefficient of variation of consecutive estimations of "migration" areas of leucocytes from individual subjects was 18 per cent with limits of 8 to 35 per cent. No effect on these areas of cell movement could be demonstrated by the addition of P.P.D. to the preparations. Gravity influenced the areas of cell movement and this is illustrated in Fig. 3a and b. The migration of mouse peritoneal macrophages from the capillary tubes was uniformly poor and the greatest area of migration ($4mm^2$) obtained is shown in Fig. 3c.

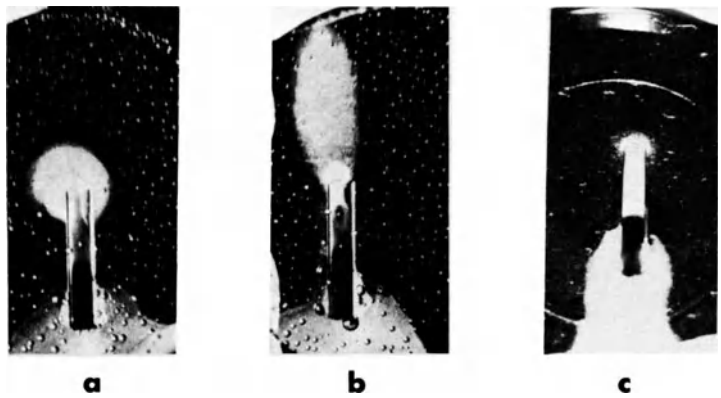

Fig. 3 (a) "Migration of human leucocytes from a capillary tube after incubation at 37°C in Eagle's medium for 16 h.

(b) Effect of gravity on a capillary tube preparation of human leucocytes. The experimental conditions were identical with those of the preparation illustrated in (a) except that the chamber was tilted at an angle of 5° from the horizontal plane along the axis of the tube and this increased the area of apparent "migration" by 35%.

(c) Migration of mouse peritoneal macrophages from a capillary tube. The preparation was incubated at 37°C for 16 h in Eagle's medium containing 10% heat-inactivated calf serum. The area occupied by the migrating cells was 4 mm^2 and the majority of the original 4 x 10^6 macrophages can be seen as a column of cells inside the capillary tube.

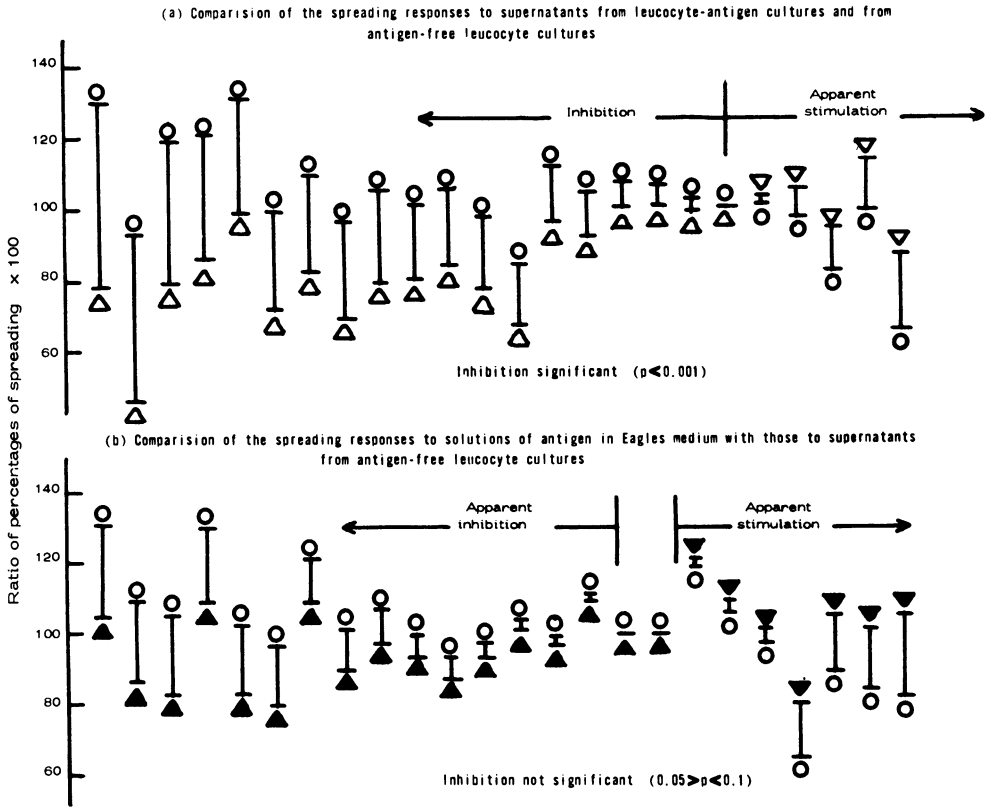

Fig. 4 (a) Inhibition of spreading of mouse macrophages on glass at 37°C by supernatants from cultures of human leucocytes and P.P.D. compared with the effect on spreading of supernatants from antigen-free leucocyte cultures.

(b) Apparent inhibition of spreading of macrophages by solutions of P.P.D. in Eagle's medium as compared with the effect on spreading of supernatants from antigen-free cultures.

▲ $\frac{\%\text{ spreading in supernatants from leucocyte-P.P.D. cultures}}{\%\text{ spreading in solutions of P.P.D. in Eagle's medium}}$

○ $\frac{\%\text{ spreading in supernatants from antigen-free cultures}}{\%\text{ spreading in Eagle's medium}}$

▲ $\frac{\%\text{ spreading in solutions of P.P.D. in Eagle's medium}}{\%\text{ spreading in Eagle's medium}}$

Inhibition of Spreading of Mouse Macrophages on Glass by Leucocyte-Antigen Reaction Products

The effect of incubation with supernatants of cultures of leucocytes and P.P.D. on the spreading of macrophages on glass was determined by comparing the ratios of percentages of macrophage spreading in test and control supernatants (Fig.4). The Wilcoxon matched pairs - signed ranks test (6) showed that supernatants from leucocyte - P.P.D. interaction significantly ($p < 0.001$) inhibited the spreading of macrophages on glass as compared with supernatants from leucocytes incubated without antigen (Fig. 4a). This test rejected the hypothesis ($0.05 > p < 0.1$) that the antigen control inhibited the spreading of macrophages as compared with the cell control (Fig. 4b). The skin sensitivity to P.P.D. was known with precision in over one third of the 24 subjects who were tested but the rank of the skin reactions did not always correspond with that of the responses of the macrophages to products of interaction between P.P.D. and the subjects' sensitized lymphocytes.

DISCUSSION

The movement of leucocytes from capillary tubes has been proposed as a technique for the measurement of cell mediated immunity in man (7) but the method has been subjected to criticism (8). Inhibition of leucocyte "migration" has none-the-less been employed to monitor the waxing and waning of lymphocyte sensitization in human organ transplantation (9, 10). High variance in results and high coefficients of variation in individual determinations were encountered in the present investigation and only dramatic inhibition of cell movement by antigen could have nullified the effects of such variation. Cell-associated fibrin formation in heparinized blood may be advanced as an explanation for the diverse results of the capillary tube experiments. Heinrich and his colleagues (11) have showed that working concentrations of heparin (6 i.u./ml) resulted in the formation of 600 µg./ml of red cell associated fibrin in heparinized blood obtained by routine venepuncture and a concentration of 450 i.u./ml was necessary to suppress the formation of this type of fibrin. The high scatter in leucocyte "migration" areas that I encountered was possibly caused by variable cell to cell adhesion in the capillaries due to cell-associated fibrin, and the same type of fibrin could also have been responsible for the low ability of mouse macrophages to migrate from capillaries. It is suggested, therefore, that all experiments involving movement of cells from capillaries could give fallacious results unless cell-associated fibrin formation is suppressed. It has been further suggested (A.E. Stuart, personal communication) that pathogen-free animals could be used as a source of macrophages in capillary tube migration experiments to lessen the possibility

of obtaining abberant results from the adhesive action of fibrin at the cell surface.

Dekaris and his co-workers (12) have reported that the spreading on glass of macrophages from sensitized animals was specifically inhibited by the corresponding antigen. It has been shown in the present investigation that spreading of unsensitized xenogeneic macrophages is inhibited by the products of reaction of sensitized lymphocytes with antigen. The skin responses of individuals of known tuberculin sensitivity, however, did not always correspond in rank with the in vitro macrophage responses although the validity of the inhibitory effects as a whole could be demonstrated statistically.

The reliability of any method of measuring cell mediated immunity in vitro depends almost entirely upon sound biological assay of lymphokine activity and this crucial step demands critical examination before results can be used with confidence. For clinical purposes, it is also necessary for a test to produce results rapidly, and this time-factor alone reduces the value of lymphocyte transformation experiments. The present investigation has called into question the validity of capillary tube leucocyte "migration" experiments as a means of measuring cell mediated immunity since the very experimental technique appears to be subject to the variably adhesive properties of cell associated fibrin; the results also suggest that fibrin at the cell surface could similarly invalidate macrophage migration experiments. A soluble "antibody-like" lymphokine that is responsible for inhibition of macrophage migration (3) and a soluble substance with mitogenic activity (2) have both been demonstrated in supernatants from cultures of sensitized lymphoid cells and antigen. The present investigation has also shown that lymphokine assay of supernatants from cultures of human leucocytes and antigen also holds promise, although the degree of inhibition of macrophage spreading by such lymphokines was not always related to the rank of the skin responses to the antigen in the corresponding subjects. It would seem, therefore, that assay of biological activity other than macrophage inhibition is worth exploring in human leucocyte-antigen culture supernatants in an attempt to find a method of measuring cell mediated immunity with a greater degree of confidence than is now possible.

SUMMARY

Evidence is presented which suggests that measurement of cell-mediated immunity by determining the inhibition by antigen of "migration"of human leucocytes from capillaries is fallacious possibly because cell-associated fibrin interferes with the free movement of leucocytes. Measurement of lymphokine activity in

supernatants from cultures of human leucocytes and antigen seems to hold promise and activity was demonstrated in such supernatants by their ability to inhibit the spreading of unsensitized xenogeneic macrophages on glass. This biological assay, however, is insufficiently precise to be used as a means of measuring cell mediated immunity because correspondence was not always achieved between inhibition of macrophage spreading and skin response to the antigen.

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IN VITRO STUDIES OF DELAYED HYPERSENSITIVITY BY THE METHOD OF MACROPHAGE SPREADING INHIBITION. DELAYED HYPERSENSITIVITY TO TUBERCULIN AND DIPHTHERIA TOXOID IN RATS.

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It was shown by early experiments (1) in mice or guinea pigs having delayed hypersensitivity, that the ability of peritoneal macrophages to spread is markedly decreased by the presence of specific antigen. The spreading inhibition can be easily determined and used as a simple in vitro test for the study of delayed hypersensitivity. The method was named macrophage spreading inhibition (MSI) and is usefully applied for the detection of delayed hypersensitivity to bacterial and protozoal antigens in guinea pigs and mice (1,2,3,4).

This report deals with the experiments in rats sensitized either to tuberculin or to diphtheria toxoid. It is shown that MSI technique can also be applied for the detection of delayed hypersensitivity in this animal species.

MATERIAL AND METHODS

The animals were tested either with tuberculin purified protein derivative (PPD RT 23, Statens Seruminstitut, Copenhagen, solution, 100 mcg per ml), or with a purified diphtheria toxoid ("Dipur 26", Imunološki zavod, Zagreb, 4200 Lf per ml).

Wistar rats (160 - 200 g) were sensitized to tuberculin either by a single injection of 0.05 ml of incomplete adjuvant (Difco Lab., Detroit) containing 3 mg of heat killed BCG (Republički zavod za zaštitu zdravlja, Zagreb) per ml or by the injection of 0.05 ml of a suspension of Mycobacterium butyricum in Freund's adjuvant (Difco) into the hind footpads. Hypersensitivity to diphtheria toxoid was produced by the injection of a saline diluted diphtheria toxoid emulsified with an equal

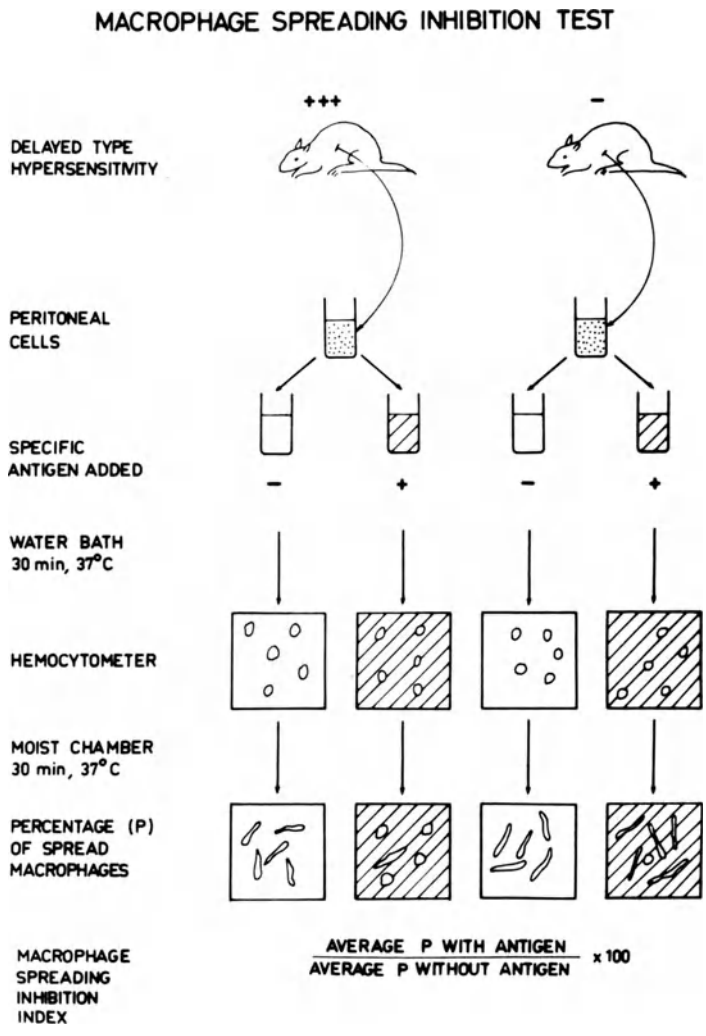

Fig. 1. Schematical presentation of the macrophage spreading inhibition test: Peritoneal cells are harvested from sensitized and control animal. The control cell suspension in tissue culture medium alone, or suspension mixed with one of the test antigens is incubated at 37°C for 30 min. After incubation the cells are resuspended and placed in a hemocytometer in a moist chamber for another 30 min at 37°C. The cells are then examined under a phase microscope and the percentage of spread macrophages is established. Since the inhibition of spreading is not complete in the presence of specific antigen, the calculated index is used for an evaluation of spreading inhibition. The lower the figure for macropahage spreading inhibition index obtained, the greater is the spreading inhibition.

volume of incomplete Freund's adjuvant (Difco). Each animal was injected into the hind footpads with 0.05 ml of the emulsion containing 50 Lf of diphtheria toxoid.

Tissue culture medium 199 (Imunološki zavod, Zagreb) containing 20 mg of bovine serum albumin and 5 units of heparin per ml was used as a control antigen and medium for cell incubation.

Macrophage spreading inhibition test was performed as follows: Peritoneal cells were harvested two to four weeks after sensitization. This was done by injecting 10 ml of medium 199 into the peritoneal cavity under the sterile precautions. Immediately after injection the syringe was removed and the injected liquid came out through the same needle into a glass tube. These and the following steps (see Fig. 1) were described in detail elsewhere (1,2,3). The peritoneal cell suspensions harvested from sensitized or normal rats were tested by mixing 0.9 ml with: i.) 0.1 ml of medium 199, ii.) 0.1 ml of medium 199 containing 5,2,1 or 0.1 mcg of tuberculin, or iii.) 0.1 ml of medium 199 containing 5.25 or 1.05 Lf of diphtheria toxoid.

RESULTS

Cell counts were performed on a sample of the peritoneal washings by a phase contrast microscope. It was found by a trypan blue dye exclusion that more than 95 per cent of cells are viable. The cell population was composed of macrophages, lymphocytes, granulocytes and mast cells. The number of erythrocytes was variable, but usually low.

In the group of twelve rats, peritoneal cells were harvested few hours before and two weeks after sensitization with BCG in adjuvant. There was no difference between the average macrophage spreading inhibition indices before and after sensitizing injection (TABLE I). Similarly, two weeks following sensitization an intradermal injection of 5 mcg of tuberculin did not provoke any measurable cutaneous reactivity either at 4 or 24 hours period.

When the rats were sensitized with Mycobacterium butyricum in adjuvant the 24 hours average skin reactions to intradermal injection of 1 or 0.1 mcg of tuberculin were notable, but weak (6.3 and 5.4 mm respectively). The results of macrophage spreading inhibition test for this group of animals are summarized in TABLE II. It can be seen, that for a high test dose of tuberculin (1 mcg per ml) there was a significant difference of the average macrophage spreading inhibition index between normal and sensitized rats ("t" test, $P<0.01$). In contrast, there was no significant difference between average macrophage spreading inhibition indices in the presence of a low test dose of tuberculin (0.1 mcg per

TABLE I. Indices of macrophage spreading inhibition by tuberculin before (day 0) and two weeks (day 14) after sensitization of rats with BCG in adjuvant

Tuberculin mcg per ml							
5		2		1		0.1	
Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14
17	21	45	81	118	49	62	121
6	53	30	97	74	96	149	106
68	62	135	133	135	175	107	82
56	50	146	42	108	82	123	84
40	71	194	88	337	118	206	104
25	45	98	122	138	134	100	136
133	63	109	108	128	130	93	133
52	104	54	101	103	117	94	67
153	55	134	92	146	86	86	83
68	77	128	62	132	112	79	108
86	43	68	86	113	128	73	86
93	66	116	90	148	101	110	78
			Average				
66	59	105	92	140	111	107	99

ml). Similar to previous group sensitized with BCG, a variability of macrophage spreading inhibition indices within each group was rather pronounced.

Another group of rats was sensitized with diphtheria toxoid in adjuvant. An intradermal injection of 42 or 10 Lf of diphtheria toxoid provokes in these animals only a weak average cutaneous reactivity (8.5 and 3.0 mm respectively). Four or twenty four hours after the challenge skin reactions were of the same average diameter, but of different aspect: edematous at 4 and indurated at 24 hours. When tested in vitro the peritoneal macrophages of these animals were significantly inhibited only with diphtheria toxoid, but not with heterologous antigen (TABLE III). If sensitized rats were compared to normal animals there was more inhibition of macrophage spreading in the presence of a high test dose of diphtheria toxoid ($P < 0.001$), than in the presence of the lower dose of toxoid ($P < 0.05$). Further, MSI indices were more uniform in the presence of the higher test dose of diphtheria toxoid. There was no spreading inhibition in the presence of a high test dose of tuberculin.

TABLE II. Indices of macrophage spreading inhibition by tuberculin in normal rats and rats sensitized with Mycobacterium butyricum in adjuvant

Normal Animals		Sensitized animals	
Tuberculin mcg per ml			
1	0.1	1	0.1
96	160	87	98
15	75	49	69
39	60	23	50
82	96	41	120
96	44	27	135
93	108	34	82
101	100	117	221
152	148	58	56
92	96	54	33
112	113	23	50
		41	120
		27	135
		34	82
Average			
88	100	47	96

TABLE III. Indices of macrophage spreading inhibition by diphtheria toxoid (5.2 or 1.0 Lf per ml) and tuberculin (1.0 mcg per ml) in normal rats and rats sensitized with diphtheria toxoid in incomplete Freund's adjuvant

Normal Animals			Sensitized Animals		
5.2 Lf	1.0 Lf	1.0 mcg	5.2 Lf	1.0 Lf	1.0 mcg
115	100	109	22	24	167
77	91	112	37	64	38
133	114	132	85	89	100
119	128	61	53	69	92
100	110	80	38	126	98
77	100	97	24	36	78
105	96	127	19	103	107
80	100	145	70	79	126
100	113	107	52	96	
79	101	97	45	85	
		Average			
98	105	107	44	77	97

DISCUSSION

It comes out from these and previous results (1,2,3) that the macrophage spreading inhibition may be considered as an in vitro correlate of a delayed hypersensitivity. With regard to this statement, a certain number of points was established or confirmed by the present study.

Firstly, the delayed hypersensitivity to bacterial antigens in rats can be detected by the MSI method. The specificity of spreading inhibiton was shown in animals sensitized to diphtheria toxoid. The MSI was only provoked with diphtheria toxoid, but not with a high test dose of heterologous antigen (tuberculin). Regarding reproducibility of this method, it was found that the variability of individual MSI indices tended to be less pronounced in the presence of higher test dose of antigen. Further, it should be noted that in animals having only the low cutaneous delayed reactivity, the MSI in the presence of specific antigen was well pronounced. The appearance of an in vitro MSI parallels the appearance of delayed skin reactivity. When sensitizing procedure does not evoke cutaneous reactivity there was also no macrophage spreading inhibition. These results and the previous observations regarding the correlation between late cutaneous reactivity and MSI test (2) indicate a relevance of the observed macrophage spreading inhibition in vitro to events occuring in vivo in delayed hypersensitivity. Finally, as for the mechanism of MSI, our initial hypothesis (1) that macrophage spreading inhibition in the presence of specific antigen occurs under similar conditions as the inhibition of migration from tissue explants or capillary tubes seems to be supported by experimental findings. First of all, it was found that animals immunized to ovalbumin in such a way to produce circulating antibodies in absence of delayed skin reactivity, showed no macrophage spreading inhibition in the presence of ovalbumin (1). Further, spreading of normal macrophages is unaffected either by incubation with antiserum prior to contact with an antigen or if antigen-antibody reaction takes place simultaneously with the process of macrophage spreading (5). Some preliminary experiments seem particularly relevant with regard to this question. Thus, if lymphocytes from guinea-pigs with a delayed-type hypersensitivity to tuberculin were cultured with tuberculin in vitro, a cell-free supernatant (supernatant A) inhibiting the spreading of normal peritoneal macrophages was obtained. A paired culture of the same lymphocyte population was made without antigen, with tuberculin added to cell-free supernatant (supernatant B) in order to reconstitute the concentration of antigen already present in supernatant A. The ratio of percentages of macrophage spreading for these two supernatants was: $A/B = 29.8$ ($P < 0.001$); i.e. there was a significant difference of the spreading percentage of normal macrophages incubated in the supernatant A compared to supernatant B. A similar effect of cell-free supernatants on macrophage spreading was reported by

T.D. Brogan on this Meeting (6). These results indicate that a soluble factor released by a sensitive lymphoid cells upon contact with an antigen is responsible for the macrophage spreading inhibition in vitro in a delayed-type hypersensitivity.

SUMMARY

It was found that the cell population of peritoneal washing of Wistar rats is composed of macrophages, lymphocytes, granulocytes, mast cells and variable number of erythrocytes. More of 95 per cent of cells were viable. The spreading of macrophages obtained from animals sensitized with BCG in the incomplete Freund's adjuvant was not inhibited by tuberculin. On the contrary tuberculin significantly inhibited the spreading of macrophages from animals sensitized with Mycobacterium butyricum in adjuvant. Macrophages obtained from animals sensitized with diphtheria toxoid in the incomplete Freund's adjuvant were significantly inhibited from spreading by diphtheria toxoid, but not by tuberculin.

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SUPPRESSION OF SECOND SET REACTION IN TRANSPLANTATION IMMUNITY BY PRETREATMENT WITH HISTONE OF GRAFT RECIPIENTS AND DONORS

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Pretreatment of allograft recipients with histones extracted from cell nuclei results in prolongation of allograft survival time (1). A similar immunodepressive effect of histone has been demonstrated for humoral antibody response (2) and for sensitization using anaphylactic and tuberculin type reactions as experimental models (3). These findings are consistent with the observation that histones inhibit DNA dependent RNA-synthesis in cell-free systems (4,5). Furthermore, it was found that allograft rejection was also delayed when only the graft donors were treated with histone (6). These results were interpreted to suggest that histone prevents the transplanted cell to some degree from being recognized as antigen ("not self") by the homologous graft recipient.

It was of interest to examine whether or not the second set reaction in transplantation immunity might be overcome by pretreating the graft recipients as well as the graft donors with histone using live spleen cells of the graft donor's line for presensitization. Skin grafts from male inbred mice of strain C57/Bl/BOM were transplanted on male inbred mice of the AKR/A/BOM strain (Laboratory Animals Breeding and Research Center, Gl.Bomholtgard, Ry, Denmark) weighing from 22 to 28 g. Transplantations were performed with punch pliers (1). The round skin grafts had a diameter of 1o mm. Second set reaction was induced in control animals by intravenous injections of 2 x $1o^7$ spleen cells of the strain of graft donors obtained by the method of Janowsky et al. (7). Total histone was extracted from cell nuclei from calf thymus using sulfuric acid of pH o.7 (1) and the histone doses were expressed in terms of histone-nitrogen (Kjeldahl).

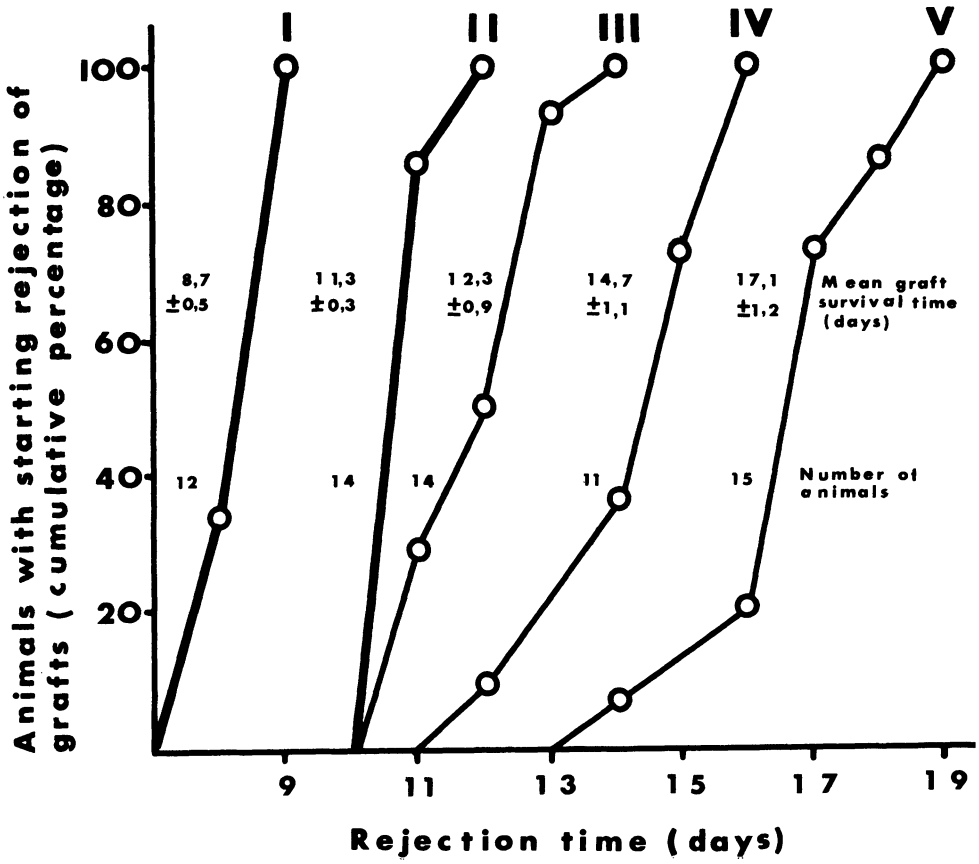

Figure 1 - Influence of histone on second set reaction in transplantation immunity. I. Presensitization of graft recipients with spleen cells of the graft donor's line (second set reaction). II. Skin grafting without presensitization and without treatment (control). III. Pretreatment of graft recipients and graft donors with histone; histone - treatment continued; no presensitization. IV. Pretreatment of graft recipients with histone; sensitization with spleen cells of donors pretreated with histone; transplantation of skin grafts from donors pretreated with histone.

When graft recipients were presensitized with spleen cells of the graft donor's line two weeks before skin grafting, an accelerated rejection of allotransplants was observed (Figure 1/I). The mean graft survival time was 8.7 days as compared to 11.3 days in the non presensitized control group (Figure 1/II). The difference in the mean survival times between these two groups is statistically significant ($P < 0.01$).

As additional control, the immunodepressive effect of histone was assayed in mice not presensitized with spleen cells. For this purpose AKR-mice were given four subcutaneous injections of histone during two weeks prior to transplantation. The individual dose was o.25 mg histone-nitrogen. Then, grafts from C57-donors pretreated 1 to 2 hours before with a single dose of 63 µg histone-nitrogen intravenously were taken. The histone treatment of graft recipients was continued giving two injections per week.

It was found (Figure 1/III) that graft rejection was significantly ($P < o.o1$) delayed in comparison to the control group not treated with histone.

In another experiment (Figure 1/IV), AKR-mice were pretreated with four injections of histone in like manner and were subsequently sensitized intravenously with spleen cells from C57-mice. One to two hours before cell harvesting, cell donors were treated intravenously with a single dose of 63 µg histone-nitrogen. Then, treatment of recipients with histone was discontinued in this group. Two weeks later, skin grafts were transplanted from normal, not pretreated mice. The mean graft survival time was 14.7 days and was significantly ($P < o.o1$) different from that in experiment I and also from that of experiment III. Therefore, these results demonstrate clearly that the second set reaction was overcome.

In experiment V it was examined whether or not these results could be improved by pretreating also the skin graft donors 1 to 2 hours before transplantation with 63 µg histone-nitrogen injected intravenously. Clearly, an additional delay in graft rejection was observed. The mean rejection time of 17.1 days differed significantly from that in experiment IV.

The main experiments (i.e. the experiments II and V; Figure 1) were repeated using a patch-technique for transplantation, where grafts were cut with scissors. The mean graft survival time was $2o.8 \pm o.9$ days in the group treated with histone and cells compared to $13.5 \pm o.6$ days in the control. The difference in the mean survival time between the two groups was statistically significant ($P < o.o1$). The results of this series of experiments agreed well with those described above. Only, the values of the control group were somewhat better than those of the corresponding controls of the first series (Figure 1). This difference could be due to the different transplantation techniques employed.

In these experiments it could be shown that second set reaction was overcome when presensitization of graft recipients was accomplished by live immunocompetent cells obtained from the inbred line of graft donors. Therefore, it is a tentative suggestion to succeed somehow in this way inducing an immunological chimerism. Experiments in this sense are in progress.

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THE DEPRESSIVE EFFECTS OF IMMUNOGLOBULIN CONTAINING FRACTIONS OF SERUM UPON ERYTHROPHAGOCYTOSIS

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One of the important functions of the macrophage system appears to be the detection and phagocytosis of foreign material. In vivo, the phagocytic reaction to foreign material is modified by the presence of chemotactic and opsonic materials contained in the body fluids. Studies of phagocytosis in vitro, in the presence of serum, are likely also to reflect merely the activity of the opsonic substances contained within the environment, and not the intrinsic capacity of the macrophage to react with foreign material.

Macrophages retain a degree of phagocytic reactivity towards certain particles in the absence of whole serum. For example Fauve (1964) demonstrated the phagocytosis of avirulent strains of S. typhimurium by macrophages maintained in a medium containing only serum albumin. There are indications that the physiological state of the macrophage is important for the promotion of phagocytosis, particularly in the absence of serum. Suter and Ramsier (1964) reported that serum opsonins were required for the phagocytosis of S. typhimurium by macrophages in suspension, but that the same organisms were readily phagocytosed by macrophages maintained as monolayers upon glass for 24 to 48 hours, in the absence of serum. Stuart (1967) recommends the use of macrophage cultures that are at least 24 hours old, and also states that the reactivity of macrophages decreases in prolonged culture.

During investigations into the physiology of phagocytosis, it was observed that mouse macrophages that had been cultured in media containing no serum proteins showed a greater degree of phagocytic reactivity towards the red cells of some animal species than did matched monolayers grown in media containing serum.

This increase in macrophage reactivity was evident only after at least six hours of culture in the absence of serum. This observation leads to the further investigation of the depressive effects of serum upon erythrophagocytosis by mouse macrophages. The preliminary experimental results are reported here.

EXPERIMENTAL TECHNIQUES AND QUANTITATION

Mouse macrophages were obtained by peritoneal lavage from mice 6-8 weeks old of Tucks T.O. strain. This is a closed colony obtained from A.R. Tuck & Son, Rayleigh, Essex. These were cultured in medium 199 (Glaxo) supplemented with mouse serum, human serum, or serum free medium supplement, in concentrations of 10% v/v. The cultures were prepared according to the technique of Stuart (1967), and were incubated at 37°C for 24 hours before use.

The serum free medium supplement used had the following composition:

Lactalbumin Hydrolysate (Difco TC grade)	4.7 gm.
Oyster glycogen (B.D.H.)	1.0 gm.
Sucrose (Analar Grade)	as required
L Glutamine (B.D.H.)	20 mg.
Calcium Pantothenate (B.D.H.)	10 mg.
Water (glass distilled)	to 100 ml.

The osmolarity of the supplement was adjusted by the addition of sucrose, using the washed human red cell as an osmometer. The storage qualities of the medium were found to be improved by the addition of polyvinyl-pyrrolidine (Koch-Light) 0.75 gm/100 ml., or by immunoelectrophoretically pure, salt poor, human serum albumin (provided by Dr. R.A. Cummings, South East Regional Blood Transfusion Service, Edinburgh) in a concentration of 0.65gm/100 ml. In the reported experiments, media supplements containing albumin or polyvinyl pyrrolidine were used in addition to the supplement as given above. No significant differences in the results were obtained. Dialysed and freeze dried fractions of lactalbumin hydrolysate were used to make up the medium.

Before experimental use, cultures were examined by light microscopy and any showing undesirable morphological features, such as a high proportion of rounded cells, large accumulations of cell debris, organisms, or dead cells were discarded. Cultures were also assessed on a functional basis by the addition of haemolysin coated human or mouse red cells and also by the addition of fresh washed mouse red cells. Normal healthy mouse macrophage cultures in medium 199 containing mouse serum, human serum, or lactalbumin supplement fail to ingest mouse red cells

(Phagocytic index of less than 1%) and ingest rabbit haemolysin coated red cells maximally (Phagocytic index greater than 90%). Cultures which failed to meet these functional requirements were considered unfit for experimental use and were discarded.

The reactivity of the cultures was quantitated using the expression P (the phagocytic index) which is given by the ratio:

$$P = \frac{\text{the number of macrophages containing one or more red cells}}{\text{The total number of macrophages counted}} \times 100$$

This expression is an index of the number of macrophages phagocytically reactive towards the test particle. The values for P quoted are the mean values obtained in four separate experimental determinations. Each single experiment used a minimum of 16 separate cultures to determine the experimental value of P. The mean values obtained on recounts differed from the mean experimental values originally obtained by less than 3% for a single observer. This method of quantitation proved to be highly reproducible, and the standard deviations from the mean values were low.

Preparation of Red Cell Suspension

Human and sheep red cells were obtained by venepuncture, guinea pig red cells by bleeding from the ear vein, and mouse red cells by bleeding from the retro-orbital plexus. All red cells were taken into a large excess of physiological saline containing heparin, 0.5 units/ml., and were washed four times in heparinised saline. They were then suspended in physiological saline and packed by centrifugation at 800xg for 15 minutes. Packed red cells were made up as a 5% suspension by volume in physiological saline and added to the appropriate culture in 0.1 ml. quantities. The red cells were allowed to interact with the macrophage monolayers for 2 hours at 37°C. The cultures were then washed in saline, fixed in methanol/acetone mixture (1/1 v/v.) and stained by Giemsa's method.

Preparation of Human and Mouse Serum Fractions

Mouse serum was obtained from pooled clotted mouse blood. The serum was sterilised by filtration, and then fractionated in 2 ml. quantities on Sephadex G 200 gel. in 0.2 M borax phosphate buffer at a pH of 8.6. Human serum obtained from outdated blood was fractionated in 60 ml. quantities of Sephadex G 200 gel. in 0.1 M phosphate buffer containing 1% butanol as bacteriostatic agent. Human serum was obtained from pooled group 0 human blood,

and from a single donor of blood group B. The effluent from the column was divided into either 10 fractions (mouse serum) or 13 fractions (human sera). These fractions were then freeze dried, reconstituted to the original volume of whole serum by the addition of distilled water, and then dialysed for 24 hours against physiological saline. The protein content of each fraction was determined by cellulose acetate electrophoresis, immunoelectrophoresis, and gel diffusion using specific antisera. Each fraction was tested for molecular size homogeneity by ultracentrifugation. The serum fractions were added in 0.1 ml. quantities to mouse macrophage monolayers grown in serum free medium. The culture was then continued for 4, 6, and 24 hours before the addition of the appropriate test particle.

The human and mouse sera used in these experiments were tested for the presence of naturally occurring haemolysins and haemagglutinins directed against the test red cells. Sera which showed agglutination or lysis of test red cells in dilutions greater than 1 in 10 were excluded from the series. Adsorbed sera were not used. In situations where human serum, or human serum fractions were used as supplements in cultures with human red cells, ABO compatible red cells were used throughout.

Results

The phagocytic response of the mouse macrophage to heterologous fresh red cells in media with and without serum is shown in Table 1.

TABLE 1

The Phagocytic Response to Heterologous Red Cells by Mouse Macrophages in Serum Free and Serum Containing Media

Species of Red Cell Donor	Phagocytic Index in		
	Serum Free Medium	Human Serum	Mouse Serum
Mouse (control)	1%	2%	1%
Guinea Pig	13%	8%	5%
Sheep	18%	1%	2%
Human	37%	5%	5.9%

The results for all particles were significant at the 1% level ($P < 0.01$). The phagocytic index obtained in the absence of serum was always greater for human red cells than for either sheep or guinea-pig red cells. Further studies were therefore undertaken, using the mouse macrophage/human red cell interaction as an experimental model for the investigation of the role of serum in the depression of erythrophagocytosis.

The Time Dependence of the Depressive Effects of Human and Mouse Serum upon Erythrophagocytosis

Macrophages removed from the mouse peritoneum, and thereafter maintained in culture in the absence of serum, show only slight phagocytic activity towards human red cells over the first 4 hours in culture. Thereafter the phagocytic activity increases, and maximal values for the phagocytic index were obtained by 24 hours in culture (Table 2). Macrophages cultured for similar periods of time in media containing mouse or human serum showed no increase in phagocytic reactivity over the first 24 hours in culture.

TABLE 2

Increase in Phagocytic Response to Human Red Cells by Mouse Macrophages in Serum Free Media with Increasing Time in Culture

Time of culture in serum free medium	2 hrs	4 hrs	6 hrs	12 hrs	24 hrs
Phagocytic Index (Fresh human red cells)	5%	11%	24%	35%	38%

Macrophages that had been cultured for 24 hours in serum free medium were washed in prewarmed medium 199 at 37°C and then placed in medium 199 containing 10% mouse serum or human serum for 10 minutes, 1 hour, 3 hours, 4 hours, 6 hours, 8 hours, and 12 hours. The monolayers were then washed once more in medium 199 at 37°C, and cultured for a further 2 hours in medium 199 containing 10% lactalbumin supplement. Fresh washed human red cells were then added, and the monolayers incubated for a further 2 hours. Control monolayers were prepared from cultures which had undergone similar manipulative treatment, but which were maintained in medium 199 and lactalbumin supplement throughout. The results obtained are shown in Table 3.

TABLE 3

Depression of the Phagocytic Response of Mouse Macrophages to Human Red Cells with Increasing Time of Contact of the Macrophages with Serum

Time	10 mins	1 hr	3 hrs	4 hrs	6 hrs	8 hrs	12 hrs	24 hrs
Phagocytic Index in mouse Serum	35%	28%	18%	12%	10%	8%	6%	6%
Phagocytic Index in human Serum	28%	24%	12%	8%	7%	8%	7%	5%

These experiments showed that initially depressed macrophages retain their depression for up to 4 hours in culture (Table 2) and that mouse and human serum require to act for 4 and 6 hours respectively in order to depress the reactivity of the mouse macrophage towards the human red cell (Table 3). Beyond these times no further depression of phagocytosis occurs. The depressed cultures react normally to both haemolysin coated red cells ($P > 90\%$) and to fresh mouse red cells ($P < 1\%$).

The Effects of Serum Concentration Upon the Depression of Erythrophagocytosis Produced by Human and Mouse Serum

Mouse macrophages cannot adequately be maintained in culture in serum concentrations of less than 1%. The effects of serum concentrations below 1% were studied by diluting the serum with the requisite volume of lactalbumin supplement, and using the serum/supplement mixture in cultures at a final supplement concentration of 10% by volume. Macrophages were cultured in serum concentrations varying from 10% v/v to 0.1% v/v. After 24 hours in culture, fresh washed human red cells were added and the cultures washed, fixed and stained after a further 2 hours incubation. The results are shown in the accompanying graph (Figure 1). There is a loss of the depression produced by serum at serum concentrations between 0.9% and 0.5%. Control cultures grown in decreasing concentrations of lactalbumin supplement show a linear decrease in phagocytic ability. This effect is entirely due to inadequate nutrition of the cultured cells, since at low supplement concentrations the uptake of haemolysin coated red cells

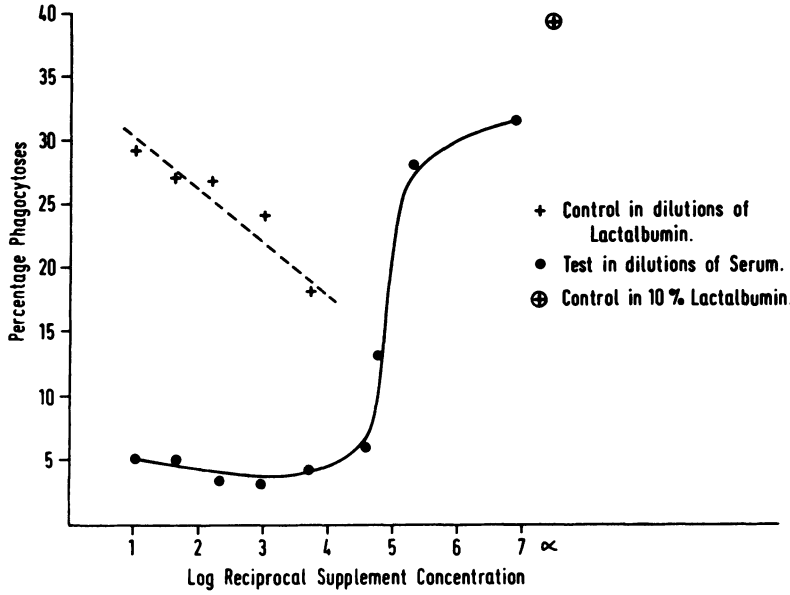

Fig. 1. The effects of serum dilution upon the phagocytic response of mouse macrophages to human red cells in vitro.

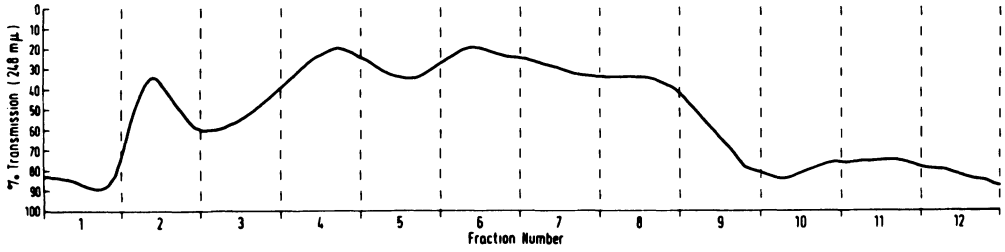

Fig. 2. Elution curve for 50 ml Gp B serum on G 200 Sephadex in 0.01 molar phosphate buffer.

is also depressed. The phagocytic activity of cells cultured in lactalbumin-serum mixtures was normal when tested with haemolysin coated red cells.

From the foregoing experiments it was established that:

(a) Mouse and human sera depress the phagocytic activity of mouse macrophage monolayers towards human, sheep and guinea-pig red cells.

(b) That the depressive effects of serum require 4 hours (human serum) or 6 hours (mouse serum) to become established, and that depression persists in the absence of serum for at least 4 hours.

(c) That the depressive effects could not be reproduced with serum concentrations of less than 0.5% by volume.

In subsequent experiments attempts were made to identify the serum components responsible for this depression. This was achieved by fractionating human and mouse serum by gel filtration, identifying the active fraction, and determining the protein content of the active fraction by immunoelectrophoresis, cellulose acetate electrophoresis, and by gel diffusion. Qualitative data only were obtained; as yet no quantitative studies have been carried out.

The Effects of Serum Fractions Upon the Phagocytosis of Human Red Cells by Mouse Macrophages

Human Serum. Human serum was obtained from outdated pooled O blood, and from a single donor of blood group B. Serum fractions were prepared as previously described. The elution curve obtained, and the relationship of the fractions to the peaks is illustrated in Figure 2.

Cultures were tested with O.Rh. negative and B.Rh. negative red cells respectively. The results obtained are illustrated in Table 4.

Phagocytic depression was observed only with fractions 4 and 5 of human serum. The depression produced by these fractions of pooled serum was significant ($P<0.01$) though less marked than that obtained with the single serum from the group B donor.

Mouse Serum. Pooled mouse serum was obtained and prepared as previously described. The column effluent was cut in such a way as to match the U.V. absorption profiles to those obtained with human serum. Fraction 4 of mouse serum corresponds to the position of fractions 4 and 5 of human serum. Ultracentrifugational studies showed these fractions to contain molecules of similar size. The effects of the mouse serum fractions upon

TABLE 4

The Depressive Effect of Human Serum Fractions Upon the Phagocytosis of Fresh Human Red Cells by Mouse Macrophages

Fraction Number	Phagocytic Index	
	Pooled Serum	Group B Serum
1	42%	40%
2	32.5%	31%
3	22.7%	25%
4	12%	5%
5	14%	12%
6	31%	31.5%
7	36%	34%
8	50%	43.75%
9	54%	54.75%
10	48%	45.75%
11	41.5%	41.25%
12	42%	42%
13	48%	54%
Controls	5.5%	3.0%
Lactalbumin Control	64.5%	64.5%

erythrophagocytosis are illustrated in Table 5. The depressive effect of mouse serum was limited to fraction 4 only.

Analysis of the Active Fractions. Fraction 4 of mouse serum studied by ultracentrifugation showed a single homogeneous peak, with an S_{20w} value of 7.043. By immunoelectrophoresis the major component was gamma-globulin, and this occupied the gamma 2 position upon cellulose acetate electrophoresis. The active fraction of mouse serum contained large amounts of 7S gamma 2 globulin. (Terminology of Fahey et al. 1964).

Fractions 4 and 5 of human serum contained several components. In each case the major component was IgG. Other proteins present in smaller quantities were alpha 1 lipoprotein, transferrin (siderophillin), and IgA. Fraction 4 on ultracentrifugation gave a single peak with an S_{20w} value of 7.146. Fraction 5 showed a major peak with an S_{20w} value of 7.04, and a secondary peak with an S_{20w} value of 5.54. On cellulose acetate electrophoresis the major component of each active fraction was gamma-globulin. As with mouse serum the active fractions contained 7S gamma-globulin (IgG). None of the other mouse or human serum fractions studied contained more than small amounts of 7S immunoglobulin, and no other fractions produced similar degrees of depression.

In order to prove that the active fractions isolated from whole serum were responsible for the depressive effects observed with whole serum, the time dependence of the phagocytic depression produced by the active fractions was studied. The fractions under study were added to macrophage monolayers which had been cultured for 24 hours in serum free medium. Incubation was continued in

TABLE 5

The Depressive Effect of Mouse Serum Fractions Upon the Phagocytosis of Fresh Human Red Cells by Mouse Macrophages

Fraction Number	Phagocytic Index in Mouse Serum
1	24%
2	21%
3	15%
4	5%
5	32%
6	38%
7	35%
Control in mouse serum	2.1%
Control in lactalbumin	42%

TABLE 6

The Time Dependence of Phagocytic Depression Induced by Human Serum Fractions

Human Serum Fractions at 4 hours	Fraction Number
40%	1
41%	2
25%	3
6%	4
11%	5
18%	6
26%	7
32%	8
56%	9
52%	10

the presence of the active fractions for 2, 4 and 6 hours. The monolayers were then washed, and the medium replaced with medium 199 supplemented with lactalbumin. The cultures were then incubated for a further 2 hours, red cells were added, and after a further 2 hours incubation the coverslips were washed, fixed and stained.

Table 6 shows that the fractions 4 and 5 of human serum produced maximal depression after 4 hours incubation with the macrophage monolayers. The degree of depression produced at this time was equivalent to that produced in a similar time period by whole serum.

Table 7 shows the depression of erythrophagocytosis produced by mouse serum fractions at 4 hours and at 6 hours. As with whole mouse serum, none of the mouse serum fractions produced depression after 4 hours. After 6 hours contact with the macrophage monolayer, fraction 4 of mouse serum, produced a degree of depression similar to that obtained by a six hour exposure to whole mouse serum.

TABLE 7

The Time Dependence of Phagocytic Depression Induced by Mouse Serum Fractions

Mouse Serum Fractions		Fraction Number
At 4 hours	At 6 hours	
32%	23%	1
30%	21%	2
26%	18%	3
22%	8%	4
41%	34%	5
40%	38%	6
36%	36%	7

DISCUSSION

There is considerable evidence that macrophages, in the absence of serum, can react with a variety of red cells. Lee and Cooper (1966) showed that the mouse macrophage in the absence of serum can react by adherence with effete homologous and heterologous red cells. Guinea pig macrophages also react with effete isologous red cells in the absence of serum (Vaughan and Boyden 1964). Rabinovitch (1967) reported that homologous and heterologous red cells modified by various chemical agents would attach to mouse peritoneal cells in the absence of serum. Perkins and Leonard (1963) showed that although mouse serum contained factors which stimulated the uptake of heterologous erythrocytes, erythrocytes from phylogenetically distant species were phagocytosed in the absence of these factors. From these and other observations, it is evident that the macrophage has an intrinsic capacity to react with effete homologous and heterologous red cells, and will react with fresh heterologous red cells in the absence of absorbable serum opsonins.

Reactivity towards effete heterologous red blood cells can be reduced by the prior treatment of the macrophage with trypsin (Rabinovitch 1968), indicating the importance of the macrophage

surface in this reaction. The actual mechanisms by which red cells attach to the macrophage surface are unknown. Vaughan (1965) suggested that the attachment of effete homologous red cells was mediated by a cytophilic factor present in normal serum. It has been demonstrated that isoantibodies directed against the human red cell A, B, and Rh antigens will cause adherence of red cells to the macrophage surface (Stuart 1967).

The experimental work described in this paper supports the conclusion that the mouse macrophage possesses an intrinsic capacity to react with at least some fresh heterologous red cells. An unexpected observation is that both mouse and human serum fractions will depress the phagocytosis of fresh heterologous red cells, and that the active fractions of both sera contain immunoglobulin.

The depressive effects of serum upon erythrophagocytosis of fresh heterologous R.B.C. have not previously been reported. This may be because these effects cannot be observed unless an experimental model is used which permits macrophages to be maintained in a serum free medium for periods in excess of 6 hours. An additional factor is the use of a system in which macrophages are cultured as monolayers upon glass. There is evidence that the phagocytic abilities of macrophages in monolayer cultures are better developed than in suspension culture (Suter and Ramsier 1964). The depression observed in the interaction between mouse macrophages and the fresh red cells from other species is not due to a generalised toxic effect of serum upon macrophages. Cultures maintained in media containing serum will maximally ingest haemolysin-coated red cells from either mouse or man. Furthermore ingestion of erythrocytes damaged by exposure to physiological saline is not impaired (Habeshaw 1970, Stuart and Habeshaw, in press). In our experiments the depression occurs with red cells which are foreign but otherwise are normal.

We are hesitant to generalise about the nature of immunoglobulin produced depression of erythrophagocytosis, particularly in the light of reported antigenic similarities between mouse peritoneal cells and human red cells (Goodman and Merchant 1954). It may be that an experimental system using mouse and human cells and serum is unique in its ability to demonstrate depression of erythrophagocytosis by immuno-globulin containing serum fractions.

The depressive effects observed are dependent both upon time of incubation of the macrophage with serum, and upon the concentration of serum in the medium. These observations indicate that the effects may be due to physical incorporation of the protein from the active fraction into the macrophage membrane. The depressive effects are also reversed by culture in the absence of serum (Table 2). The macrophage membrane appears to bear

receptor sites for IgG (Lo Buglio et al. 1967), (Huber and Fudenberg 1968), and it has been shown that this receptor resembles that for cytophilic antibody (Berken and Benacerraf 1966). In addition, it has been demonstrated that binding of red cells sensitised with IgG anti-Rh_o antibody by macrophages could be inhibited by the presence of excess free antibody in the medium (Huber, Douglas and Fudenberg 1969).

The experimental data reported in this paper could be interpreted by assuming that the intrinsic capacity of the macrophage to react with heterologous red cells is conferred by a cytophilic antibody. A loss of the capacity to react with the heterologous red cell would follow substitution of the cytophilic antibody with immuno-globulin in serum. In this sense the depressive effects are interpreted as a "competitive inhibition" of cytophilic antibody by free immuno-globulin. There is some experimental evidence that such reactions do occur (Jonas, Gurner, Nelson and Coombs 1965). The evidence presented here favours the concept that occlusion of the reactive sites on the membrane occurs by a reaction with free immuno-globulin, since the initial depression of phagocytosis observed is gradually lost over the first six hours in culture in the absence of serum, with a re-emergence of the capacity of the macrophage to ingest fresh human red cells.

At a time when there is growing evidence that lymphocytes react specifically with antigen by virtue of immuno-globulin molecules upon their surfaces it is tempting to postulate that similar mechanisms are operative in determining the reactivity of macrophages to antigen. It is feasible that immuno-globulin molecules of a cytophilic type initiate reactions of both lymphocyte and macrophage with components that are not-self. On the other hand, circulating antibodies, having a capacity to bind only with the macrophage membrane (Huber et al. 1969) can temporarily modify the reaction between macrophage and antigen in a complex manner, enhancing the uptake of antigen when specific antigen-antibody complexes of high affinity are formed in regions of antigen excess, and depressing antigen uptake when excess free antibody is present. These observations suggest a close functional co-operation between lymphoid cells and reticulo-endothelial cells particularly in regard to the homeostatic mechanisms controlling the immune response.

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ROLE OF RES IN THE DEFENSE AGAINST INVASION BY NEOPLASTIC, BACTERIAL AND IMMUNOCOMPETENT CELLS

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The principal aim of this paper is to emphasize that macrophages of the reticuloendothelial system (RES) play an important role in the mechanism of natural defense against aggression by bacterial, neoplastic or even normal cells. It has been observed that the phagocytic activity of the RES as measured by the rate of clearance of colloidal carbon from the blood (1 , 2) and expressed by the phagocytic index K is increased in all these conditions. Some examples of the RES response to different infections are shown in Fig. 1

Virulent Salmonella enteritidis infection in mice following i.v. injection produced a slight stimulation of phagocytic activity (Fig. 1A). The oral administration of the same strain of Salmonella led to a less severe infection as shown by the lower mortality rate and a greater increase in the phagocytic index K (3). This inverse relationship between virulence of infection and intensity of macrophage stimulation is also evident in the case of infection with Mycobacterium tuberculosis (4). The RES response after infection with the virulent M. tuberculosis strain, $H_{37}Rv$, is weak, whereas administration of the avirulent strain BCG produces a strong and lasting stimulation of phagocytic activity (Fig. 1B). The active substance concerned is localized in the cell wall of Mycobacterium. Fig. 1D shows the stimulatory effect of an extract from cell walls of the avirulent strain of M. phlei (9). A similar effect is produced by a methanol extract of M. tuberculosis (7). Killed bacteria (Salmonella typhi) or endotoxin from S. typhi, E. coli or S. abortus equi provoked a rise in phagocytic activity after a transient period of depression (5, 6, 7, 8). Corynebacterium parvum is another bacterial species which has been shown to have

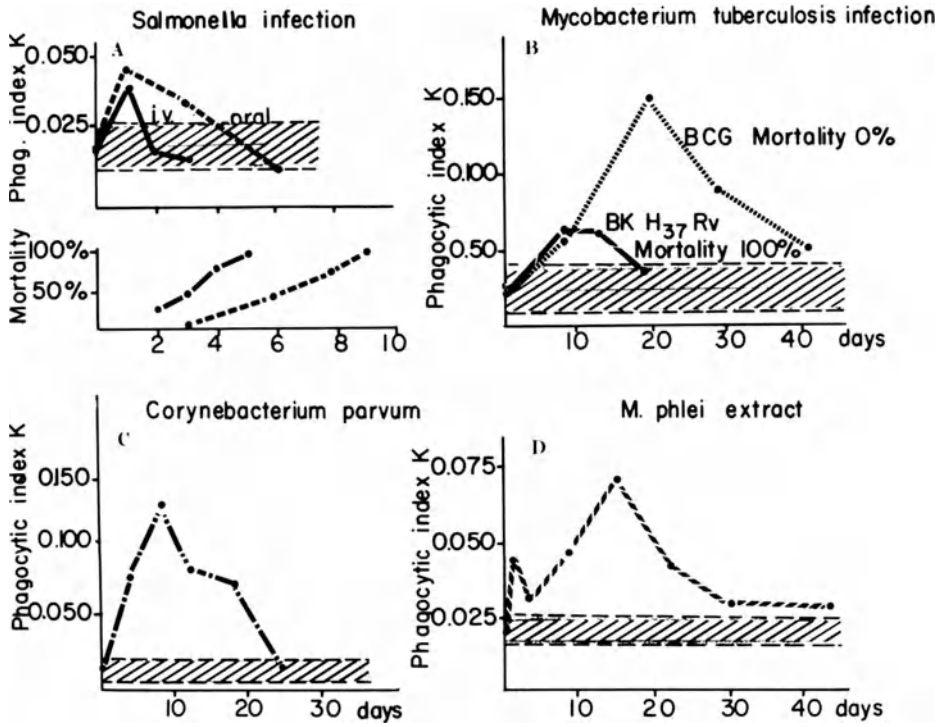

Figure 1 : Variations in macrophage phagocytic activity after injection of live or killed bacteria.

A = Infection with live S. enteritidis var Danysz : injection of 5.10^7 organisms i.v. administration of 10^{10} organisms orally.

B = Infection with live Mycobacterium tuberculosis i.v. injection of 0.05 mg (wet weight) of strain $H_{37}Rv$ or 1 mg (wet weight) of BCG.

C = i.v. injection of 500 µg killed Corynebacterium parvum.

D = i.v. injection of 400 µg of isolated cell walls of Mycobacterium phlei.

The phagocytic index K is measured by the rate of blood clearance of colloidal carbon in a dose of 16 mg per 100 g given i.v.

a potent stimulatory effect on phagocytic activity (Fig. 1C) (10).

In the case of allogeneic neoplastic cells grafted in mice, a correlation between virulence of the tumor and intensity of RES stimulation is also observed.

Betz sarcoma J was grafted into selected strains of mice endowed with different degrees of natural resistance against this tumor (Fig. 2) $C_{57}Bl_6$ mice (from which the tumor originated) were the most resistant strain, since tumor regression occured in 40 per cent of the cases. C_3H mice were rather less resistant and all the grafted mice died. F_1 hybrids of these two strains were very susceptible to the tumor with accelerated tumor growth and 100 per cent mortality. Phagocytic activity during the growth phase of sarcoma J was found to be increased in $C_{57}Bl_6$ mice, unchanged in C_3H mice and diminished in hybrid mice (11) (Fig. 2).

Another illustration of the relationship between the RES response and resistance to tumor invasion is presented in figure 3. When AKR leukemic cells are injected into syngeneic AKR mice, they rapidly proliferate and kill the host. Only a decrease in the phagocytic index K is observed in the terminal phase of the illness (Fig. 3B). On the contrary when leukemic cells are transmitted into allogeneic mice (DBA/2 leukemic cells into $C_{57}Bl_6$ mice), they are rejected after a few days of proliferation and all the mice survive. The rejection of leukemic cells is associated with a considerable rise in macrophage phagocytic activity (Fig. 3A) (12). Stimulation of phagocytic activity has also been found in mice grafted with sarcoma 180 and in mice injected with Friend's virus which induces a reticulum cell leukemia (13).

Similarly, Howard (14) has shown that there is a marked stimulation of phagocytic activity during the graft-versus-host reaction induced by injection of parental spleen cells into F_1 hybrids.

From these few examples we can conclude that, during the invasion of an organism by bacterial, tumor or normal immunocompetent cells, macrophage phagocytic activity is greatly increased. If this response denotes a defense mechanism against cell proliferation it was thought that increased protection would be provided by prestimulation of the RES. The following experiments substantiated this hypothesis.

Mice pretreated with BCG or *M. phlei* cell walls showed an increase in mean survival time after infection with a virulent *Salmonella enteritidis* strain (Table I). The protective effect was even greater when measured against infection produced by a less virulent *S. enteritidis* strain, 50 to 60 per cent of the treated mice surviving the infection (9,15).

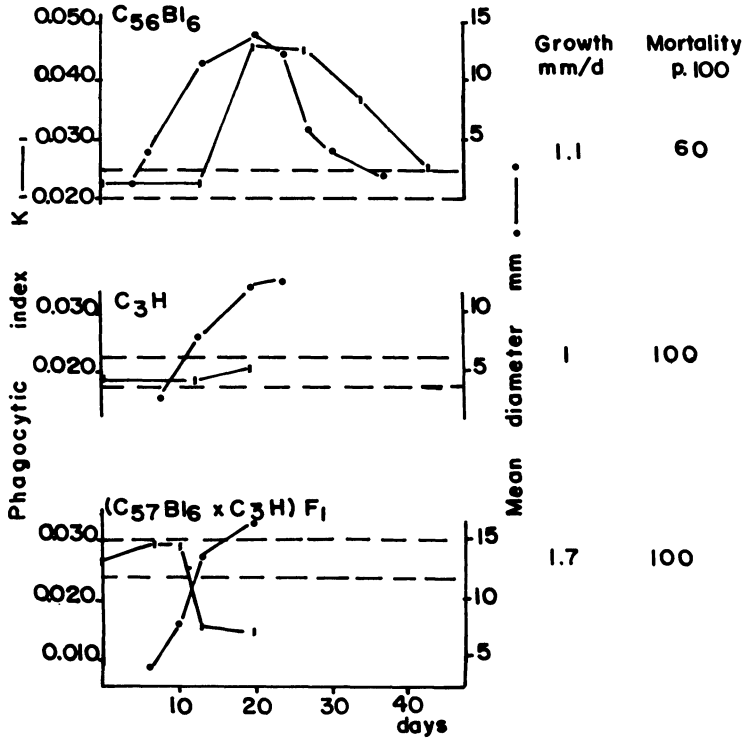

Figure 2 : Variations in macrophage phagocytic activity after a graft of Sarcoma J in different strains of mice.

The phagocytic index K is measured by the rate of blood clearance of colloidal carbon (8mg per 100g) given i.v.

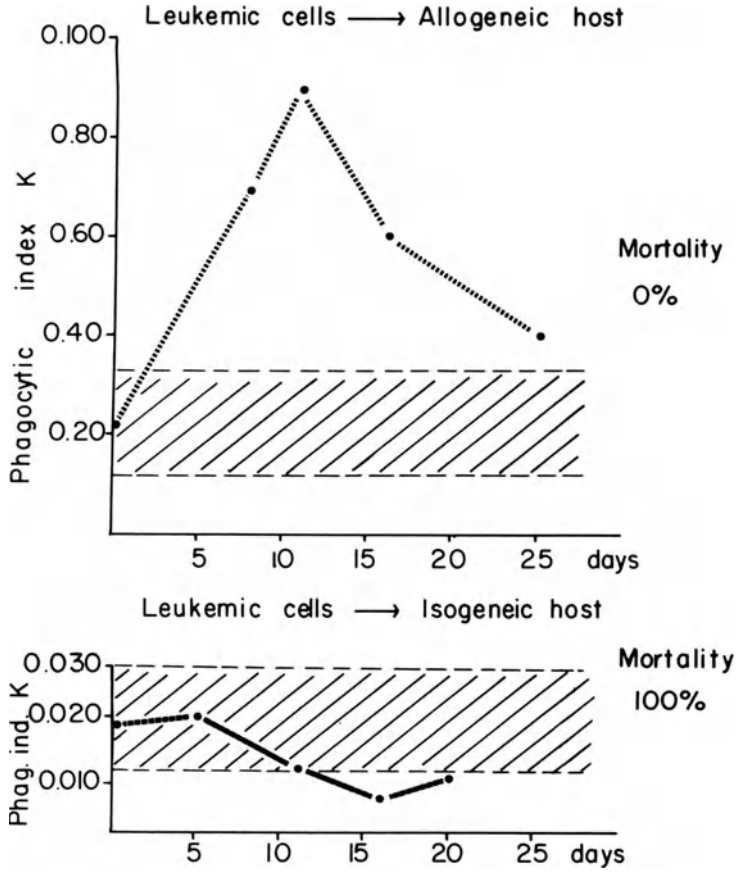

Figure 3 : Modifications of macrophage phagocytic activity after injection of allogeneic or syngeneic leukemic cells.

A = Injection of 10^6 spleen cells from DBA/2 mice (leukemia 1210) into $C_{57}Bl_6$ mice.

B = Injection of 6.10^6 lymph node cells from 8 month-old AKR mice already leukemic, into 2 month-old AKR mice.

The phagocytic index K is measured by the rate of blood clearance of colloidal carbon 8 mg per 100 g given i.v.

TABLE I

Effect of pretreatment with BCG or M. phlei on mortality in mice infected with different strains of S. enteritidis.

	Virulent strain		Attenuated strain	
	Mortality (per cent)	Mean survival time (days)	Mortality (per cent)	Mean survival time (days)
Control	100	5.1	100	7.6
BCG	100	14.2	40	> 25
M. phlei	80	25	50	> 25

BCG : i.v. injection of 1 mg (wet weight) live BCG 8 days before infection with 1000 Salmonella enteritidis

M.phlei : i.v. injection of 400 µg of isolated cell walls from M. phlei 8 days before infection with 1000 Salmonella enteritidis.

It has been shown that the rise in non specific resistance to infection is not related to enhanced specific immunization against the infecting bacteria, itself due to the strong adjuvant effect of Mycobacteria. Thus splenectomized mice show no antibody synthesis against S. enteritidis, but treated with BCG are as resistant to infection as non splenectomized-treated mice (16). Furthermore, the increase in phagocytosis is not the main factor involved in enhanced resistance, for some substances which stimulate phagocytosis powerfully have no effect on the resistance to infection (17). Rather, it is the increase in bactericidal activity of the macrophages which is chiefly concerned (7, 16, 18-25).

The effect of pretreatment with BCG or C. parvum is also evident on tumor growth (13, 26-28). The data presented in Figure 4 show the results obtained after the graft of sarcoma J in Swiss mice. Reduction in the rate of tumor growth and decrease in mortality is observed in mice treated with BCG, M. phlei or C. parvum. The most potent effect is obtained in C. parvum treated mice, 60 per cent of regression being observed (11, 29).

A similar protection can also be obtained when tumors are grafted into pretreated syngeneic hosts (26, 30-33). When 1000 or 100 AKR leukemic cells are injected into AKR mice, all the animals die. A low percentage of mice pretreated with live or killed BCG or with C. parvum survive after injection of 1000 leukemic cells. Survival rates are higher when fewer leukemic cells are injected (Table II) (34, 35).

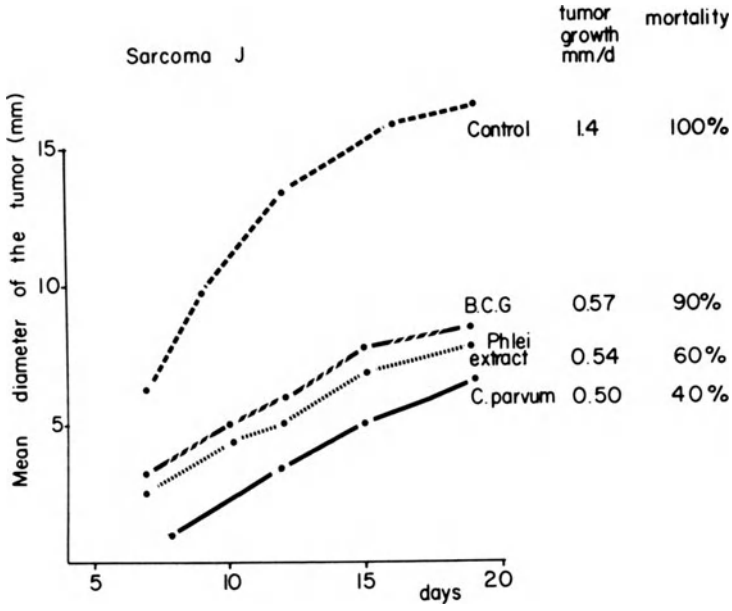

Figure 4 : Effect of pretreatment with BCG, M. phlei or C. parvum on tumor growth and mortality rate in Swiss mice grafted with Sarcoma J.

BCG : i.v. injection of 1 mg live BCG (wet weight) 11 days before sarcoma J graft.

M. phlei : Repeated injection of 400 µg of isolated cell walls of M. phlei, i.v. once a week.

C. parvum : i.v. injection of 250 µg of killed bacteria 2 days before tumor graft.

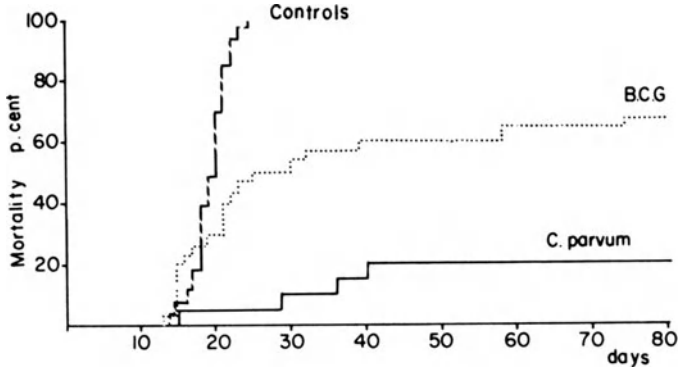

Figure 5 : Effect of pretreatment with BCG or C. parvum on the mortality from graft-versus-host disease induced in $(C_{57}Bl_6 \times C_3H)F_1$ mice by injection of 10^8 $C_{57}Bl_6$ spleen cells.

TABLE II

Effect of pretreatment with BCG or C. parvum on mortality in AKR mice injected i.p. with AKR leukemic cells (spleen cells)

	Per cent survival	
	1000 AKR cells	100 AKR cells
Control	0	0
Live BCG	30	70
Killed BCG	20	70
C. parvum	50	50

Live BCG : i.p. injection of 1 mg (wet weight), 12 days before injection of leukemic cells.
Killed BCG : i.p. injection of 2,5 mg (wet weight), 12 days before leukemia.
C.parvum : i.p. injection of 1 mg (wet weight), 7 days before AKR cells.

(From Lamensans et al., 1968 (34) (35)).

An increase in resistance against GVH is also produced by pretreatment of hybrids with BCG or C. parvum. Figure 5 shows the cumulative mortality of $(C_{57}Bl_6 \times C_3H)F_1$ hybrids injected with $C_{57}Bl_6$ spleen cells. A significant proportion of hybrids pretreated with BCG survive. The protection conferred by C. parvum is even greater (36) (37).

A possible explanation for this BCG or C. parvum induced increase in host resistance to tumor growth or GVH disease could be that extensive proliferation of lympho-reticular cells leads to enhancement of allogeneic inhibition.

In conclusion it may be said that invasion of the host by bacterial, neoplastic or normal immuno-competent cells provokes in each instance a similar macrophage response. Increased phagocytic activity is only one indication of the overall changes in macrophage function which participate in defense mechanisms. Hence, the stimulation of macrophage function by appropriate substances produces an increase in the total resistance against invading cells.

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PROTECTIVE EFFECT OF RETICULOENDOTHELIAL SYSTEM STIMULANTS IN COMBINATION WITH CHLOROQUINE ON PLASMODIUM BERGHEI INFECTION IN MICE*

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ABSTRACT

The importance of the reticuloendothelial system (RES) in the case of protozoan infections and particularly in malaria has long been recognized but never fully demonstrated. Recently, it was shown that stimulation of the RES alone is unable to increase resistance in mice against Plasmodium berghei infection.

Based on an assumption expressed earlier that the therapeutic effect of antimalarial drugs is not only directed against the malarial parasites but also requires participation of the RES, we attempted to demonstrate this relationship by using a combination of an antimalarial drug (Aralen-chloroquine hydrochloride) and an RES-stimulating agent (cholesteryl oleate, glucan or coenzyme Q_{10}).

The results of these studies show that pretreatment with low doses of chloroquine combined with RES-stimulating agents results in increased survivorship, prolonged survival time and reduced parasitemia in blood-transferred Plasmodium berghei infection in mice. The described effects were strongly dependent on the dose and the time of administration.

INTRODUCTION

The malarial problem is as actual today as it was fifty years ago despite the number of antimalarial drugs available and the better understanding of the role of the host in this disease.

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The concept that the reticuloendothelial system (RES) plays a fundamental role in resistance to protozoan infection and particularly in malaria has been the subject of much research [reviewed by Goble, et al (1)]. There is a great amount of data available on the course of malaria infection after depression of the RES, but surprisingly little is known of events occurring during stimulation. It has been shown (1-7) that after blockade of the RES induced by carbon ink, trypan blue, thorium dioxide (thorotrast), X-irradiation, etc., the parasites appear sooner and deaths occur earlier. In contrast, only certain nonspecific RES-stimulatory agents (bacterial endotoxin, nonviable Corynebacterium parvum, Freund's adjuvant) produce increased resistance to experimental malaria (8-11). Attempts to change the course of malaria by using other nonspecific RES-stimulating agents (zymosan, stilbesterol) have failed (11).

Surprisingly early, Kritchewski, et al (3), studying the effect of chemotherapy on various infections including malaria, concluded that the drug action depends on the integrity of the RES of the host. This idea was expressed again much later by Goble, et al (1), but was never fully demonstrated. Based on this assumption, we attempted in the present study to establish the significance of enhanced RES activity in combination with an antimalarial drug (Aralen-chloroquine hydrochloride) on blood-transferred Plasmodium berghei infection in mice. As RES-stimulating agents, we used cholesteryl oleate, glucan, or coenzyme Q_{10}. We demonstrated earlier (12) that intravenously administered emulsions of coenzyme Q_{10} (Fig. 1) can increase the phagocytic activity in rats as measured by the carbon clearance technique and also can increase the primary hemolytic antibody formation in mice. In addition, coenzyme Q_{10} produces no significant hyperplastic effect on the RES.

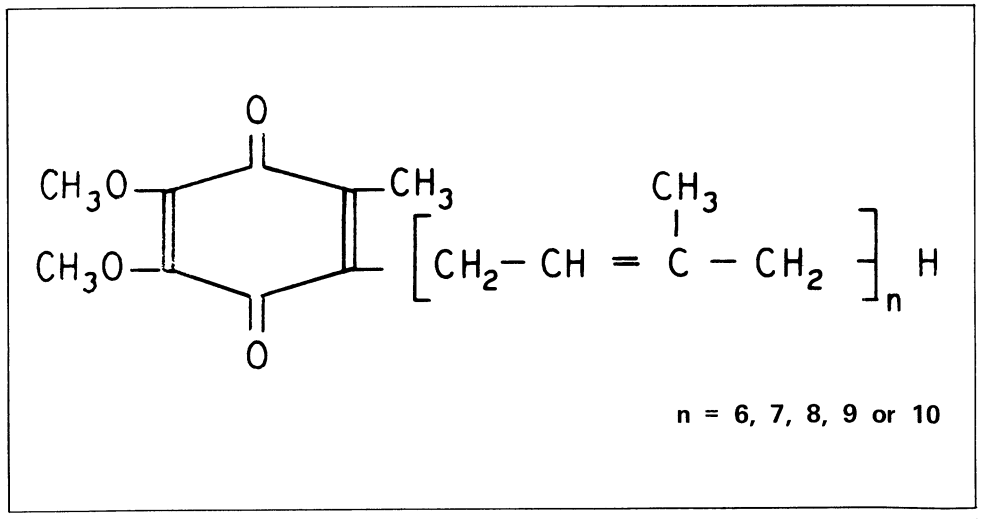

Fig. 1. Coenzyme Q or Ubiquinone.

MATERIALS AND METHODS

Animals

Male CF-1 mice, free from Eperythrozoan coccoides infection, weighing 20 g, were obtained from a single commercial breeder (Carworth Farms, Inc., New City, New York). They were housed in air-conditioned quarters, artificially illuminated during daylight hours and given food and water ad libitum. After the infection, the mortality rate was recorded for 40 days.

Infection

The New York University strain of Plasmodium berghei (NK-65), courtesy of Dr. Meir Yoeli, Department of Preventive Medicine, New York University, New York, New York, was maintained by weekly blood passages in CF-1 mice. The inoculum for each experiment was prepared by bleeding five infected mice from the retro-orbital venous plexus with five heparinized glass capillary tubes for each mouse. The pooled blood was collected in 2.5 ml sodium chloride solution (normal saline), kept in an ice bath, diluted 1:1000 in normal saline, and 0.2 ml was injected intraperitoneally. In preliminary experiments it was established that this dilution induced mortality higher than 90% within 12-16 days. These parameters were followed:

1. Per cent mortality,
2. Mean survival time,
3. Per cent parasitemia.

Parasitemia

Tail blood from animals of all groups was obtained for parasitemia determination on days 7 and 9 after the infection with Plasmodium berghei. Thin-film blood smears were made and stained with Giemsa stain. Counts of parasitized erythrocytes were based on 5000 red blood cells per experimental group obtained from 10 mice (500 RBC per mouse) and were expressed as a percentage of the control (Chloroquine-injected group).

RES-Stimulation

Cholesteryl oleate (Eastman Organic Chemicals, Rochester, New York) emulsion (30 mg/ml) was prepared in 5% glucose containing 1% Tween 20 (polyoxyethylene sorbital monolaurate) as an emulsifier. The emulsion was prepared in a Waring blender and kept in a water bath at 60°C. After homogenization for two minutes, 0.2 ml (6 mg/mouse) was injected intravenously.

Glucan (Standard Brands, Inc., Stamford, Connecticut, 1 mg/ml) was suspended in normal saline by boiling in a water bath for one hour. The suspension was prepared in a Waring blender and kept in a water bath at 60°C after addition of 0.05% Tween 20. After homogenization for 45 seconds, 0.4 ml (400 µg/mouse) was injected intravenously.

Coenzyme Q_{10} (Sigma Chemical Co., St. Louis, Missouri) emulsion (250 µg/ml) was prepared in 5% glucose containing 0.4% Tween 20. The emulsion was prepared in a Waring blender, kept in a water bath at 60°C and protected from light. After homogenization for 45 seconds, 0.6 ml (150 µg/mouse) was injected intravenously or 2 ml (500 µg/mouse) subcutaneously.

All control animals were injected with the same mixtures with the cholesteryl oleate, glucan, or coenzyme Q_{10} omitted.

The materials to be tested were administered 48 hours before the infection with Plasmodium berghei. The subcutaneous injection of coenzyme Q_{10} was administered 72 hours before the infection.

All emulsions or suspensions were tested for the presence of pyrogenicity to preclude contamination with bacterial endotoxin. U. S. Pharmacopeia-recommended criteria were used for evaluation. Only non-pyrogenic materials were used in these studies.

Chloroquine

Aralen (chloroquine hydrochloride, Winthrop Laboratories, New York, New York) was diluted in normal saline to a concentration of 3.2 mg/ml expressed as base, and 0.1 ml (320 µg/mouse) was injected intramuscularly two hours before infection with Plasmodium berghei.

General

All glassware was heated for four hours at 170°C. Non-pyrogenic Tween 20 (Sigma Chemical Co., St. Louis, Missouri), sterile 5% glucose, and sterile normal saline (Baxter Laboratories, Morton Grove, Illinois) were used.

In each experiment a minimum of 20 mice per group was used. Each experiment was repeated at least three times. The data presented in this report represent the results of a single experiment. Results of this experiment, as well as the cumulative data of all experiments, were used for statistical analysis as noted in the text.

The data obtained were statistically analyzed using the chi-square test or Student's T-test as applicable.

RESULTS

The data demonstrate that there was no significant modification of Plasmodium berghei infection in mice pretreated intravenously only with an RES-stimulating agent -- cholesteryl oleate, glucan, or coenzyme Q_{10}. Mice pretreated intramuscularly with chloroquine alone showed a slight enhancement of resistance. Increased resistance became significant when a combination of RES-stimulating agents and chloroquine was used.

The following effects were demonstrated:

1. Increase of survivorship (Figs. 2, 3 and 4),
2. Increase of survival time of the mice which died during the observation period (Fig. 5),
3. Decrease of the parasitemia (Fig. 6).

The statistical analysis showed that the results and the conclusions obtained are significant.

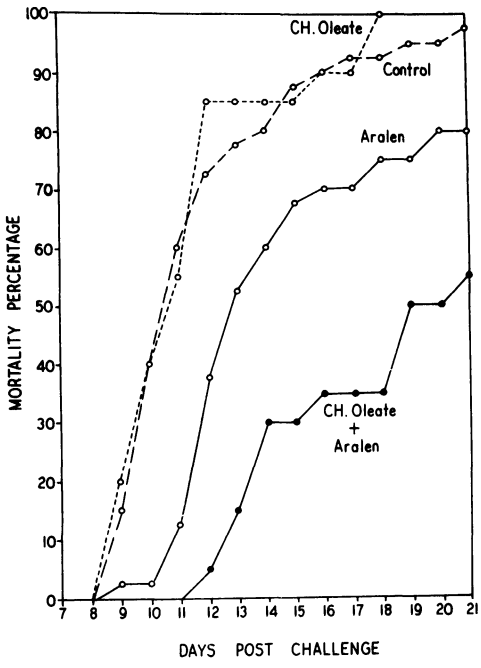

Fig. 2. Modification of mortality rate in mice infected with *Plasmodium berghei* pretreated intravenously with cholesteryl oleate (6 mg/mouse) and Aralen (chloroquine hydrochloride).

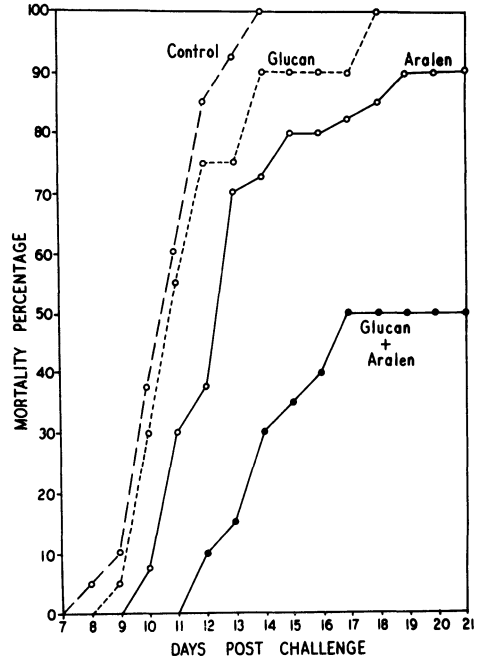

Fig. 3. Modification of mortality rate in mice infected with *Plasmodium berghei* pretreated intravenously with glucan (400 μg/mouse) and Aralen (chloroquine hydrochloride).

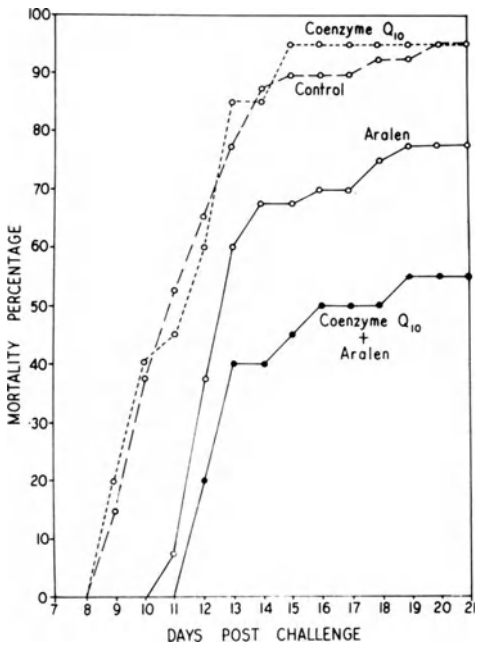

Fig. 4. Modification of mortality rate in mice infected with *Plasmodium berghei* pretreated intravenously with coenzyme Q_{10} (150 μg/mouse) and Aralen (chloroquine hydrochloride).

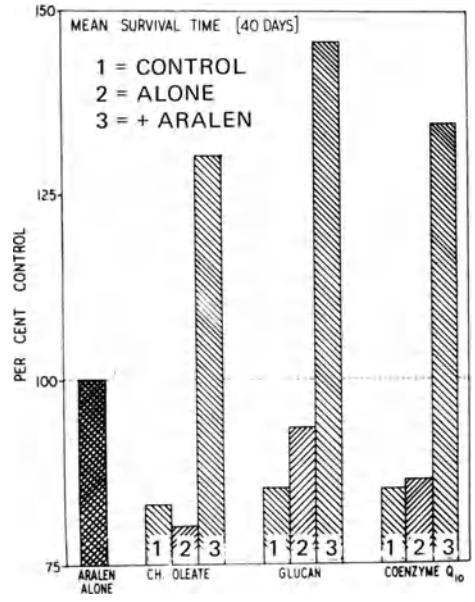

Fig. 5. Modification of mean survival time in mice infected with *Plasmodium berghei* pretreated with cholesteryl oleate (6 mg/mouse), glucan (400 μg/mouse) or coenzyme Q_{10} (150 μg/mouse) intravenously and Aralen (chloroquine hydrochloride).

Experiments using emulsions of coenzyme Q_{10} showed that the intravenous route can be replaced effectively by the subcutaneous route (Fig. 7).

Additional results not presented here demonstrated that the effects described were strongly dependent on the dose and the time of administration.

DISCUSSION

Even now, the mode of action of most antimalarial drugs is not completely understood. It was shown (13) that chloroquine inhibits the incorporation of P^{32}-labelled phosphate into RNA and DNA by the malarial parasite, thus interrupting the erythrocytic stage of its development.

Both cellular and humoral elements and events are involved in the defense against malarial infection.

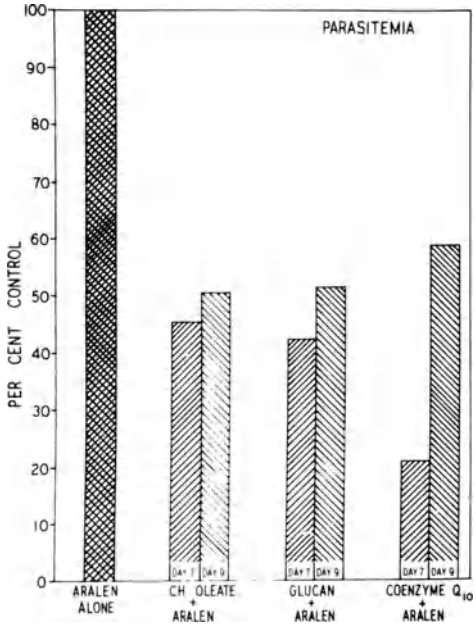

Fig. 6. Modification of percentage parasitemia in mice infected with *Plasmodium berghei* pretreated with cholesteryl oleate (6 mg/mouse), glucan (400 μg/mouse) or coenzyme Q_{10} (150 μg/mouse) intravenously and Aralen (chloroquine hydrochloride).

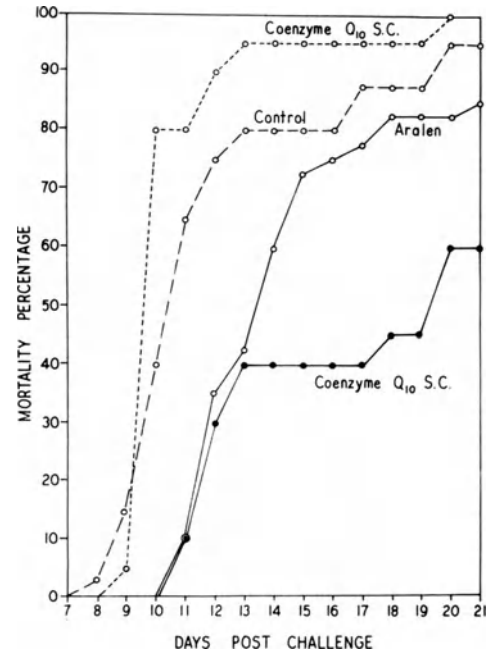

Fig. 7. Modification of mortality rate in mice infected with *Plasmodium berghei* pretreated subcutaneously with coenzyme Q_{10} (500 μg/mouse) and Aralen (chloroquine hydrochloride).

Most investigators consider phagocytosis the most important factor in removing parasites (14-17) or infected red blood cells [reviewed by Zuckerman (18)] from the circulation by the RES.

Malarial infection is followed by increased immunoglobulin synthesis and the production of weak antimalarial antibodies [reviewed by Yoeli (8)]. The immunity to malarial infection is largely species specific, and the correlation between the immune status of an individual and the observed levels of malarial antibodies is poor, which indicates that much of the antimalarial immunoglobulin formed has no protective effect against plasmodial infection (19). However, passive transfer tests have demonstrated that serum antibody does play a part in malarial immunity (8,20,21). It had been suggested (22) that specific antibody can damage parasites directly or opsonize parasitized cells preparatory to phagocytosis. More recently, it has been shown (23) that in the presence of immune serum, malarial parasites inside blood cells grow and differentiate, but re-invasion of erythrocytes is prevented and the succeeding cycle of parasite development is inhibited completely.

Our results demonstrate that weak resistance to Plasmodium berghei infection in mice produced by chloroquine may be enhanced further by the use of RES-stimulating agents. The most reasonable explanation of our results is that the increased clearance of parasites and infected red blood cells during the early stages of infection, in addition to the depressed intracellular development of the parasites produced by chloroquine, could be responsible for the retardation and, in some cases, the elimination of the infection. Later, during the infection, these events are enforced by the presence of an increased amount of specific antibody.

Further studies are in progress using a therapeutic rather than a prophylactic approach.

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THE ANTILEPROSY AGENT CLOFAZIMINE (B.663) IN MACROPHAGES: LIGHT, ELECTRON MICROSCOPE AND FUNCTION STUDIES

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In 1957 in a communication from these laboratories the remarkable antimycobacterial activity of B.663, 3-(p-chloroanilino)-10-(p-chlorophenyl)-2,10-dihydro-2-(isopropylimino)-phenazine, was first described (1). B.663, now known as Clofazimine, forms the active component of the antileprosy preparation Lamprene (Geigy).

A characteristic feature of B.663 and related compounds is that they are concentrated, in various degrees depending on their structure, within cells of the reticuloendothelial system and it was this feature which led us to suggest that they might "have an important role in the prophylaxis and treatment of tuberculosis and leprosy" (2). This "important role" in the treatment of leprosy is now well established for B.663, thanks to the pioneer work of Browne and Hogerzeil (3,4) and in the treatment of Buruli ulcer by the work of Lunn and Rees (5). The developmental work has been reviewed by Barry and Conalty (6) and Barry (7). The accumulation of B.663 within macrophages of mice following oral administration, and its effects, form the subject of the present communication.

MORPHOLOGICAL APPEARANCES

Light Microscope Findings.

The pattern of concentration of B.663 and related compounds in cells of the reticuloendothelial system,

as seen by the light microscope, has been published in detail previously (8-11), but will be repeated in outline here to facilitate correlation with the electron microscope findings to be reported now.

Briefly, the sequence of concentration in macrophages is readily followed in unstained organ smears from mice treated with B.663, 50 mg/kg daily - drug in diet. Initially macrophages are seen to contain orange-red rounded inclusion bodies or phagosomes. Macrophages throughout the body are involved but the actual time interval at which these drug-containing phagosomes are first seen varies according to the organ. As administration of the compound is continued the phagosomes darken in colour and within many of them areas of greater density appear. This pure phagosomal or phagolysosomal stage is followed within days by the appearance of bright-red crystals together with the phagosomes (Fig.1,A).

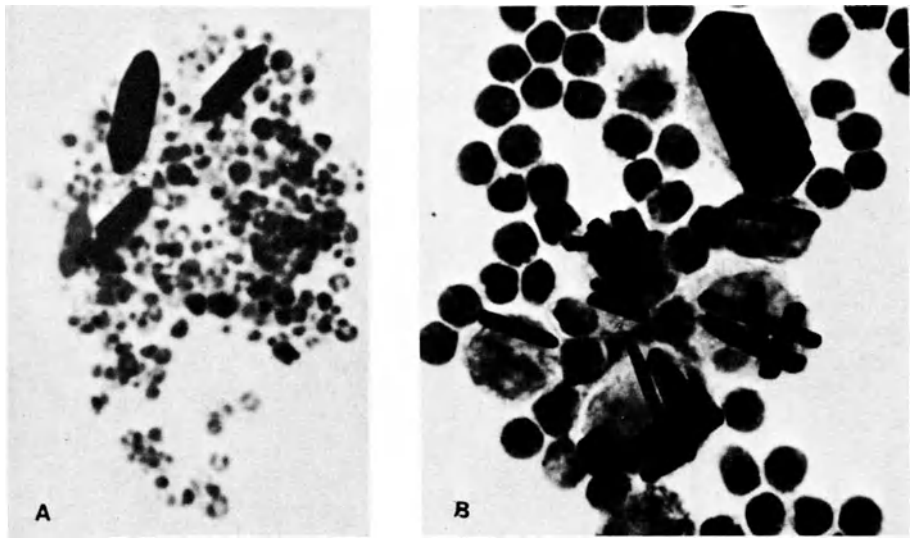

Fig.1. Accumulation of B.663 in Macrophages in Mice.

A. Lung smear. B.663-containing phagosomes and early crystal formation. Unstained. B.663 at 50 mg/kg/day for 14 days, in diet.

B. Peritoneal smear showing variation in size and number of B.663 crystals. Toluidine blue stain. Treatment for 26 days as in A.

Gradually, as treatment is continued, such cells come to contain crystals only (Fig.1,B).

Electron Microscope Findings.

Methods of fixation or staining employing alcohol, acids or lipid solvents remove the coloured drug leaving the "ghosts" of the phagosomes or crystals behind (10). Osmium tetroxide, which fixes the phagosomal bodies although crystals are dissolved out, has permitted the following interesting electron-microscope results. Corresponding to the coloured inclusions seen by means of the light microscope are electron-dense bodies varying in size and in electron density (Fig.2). In some instances, fusion of dense bodies with each other and with less dense bodies may be observed and would appear to correspond to the formation of phagolysosomes as described by de Duve (12)

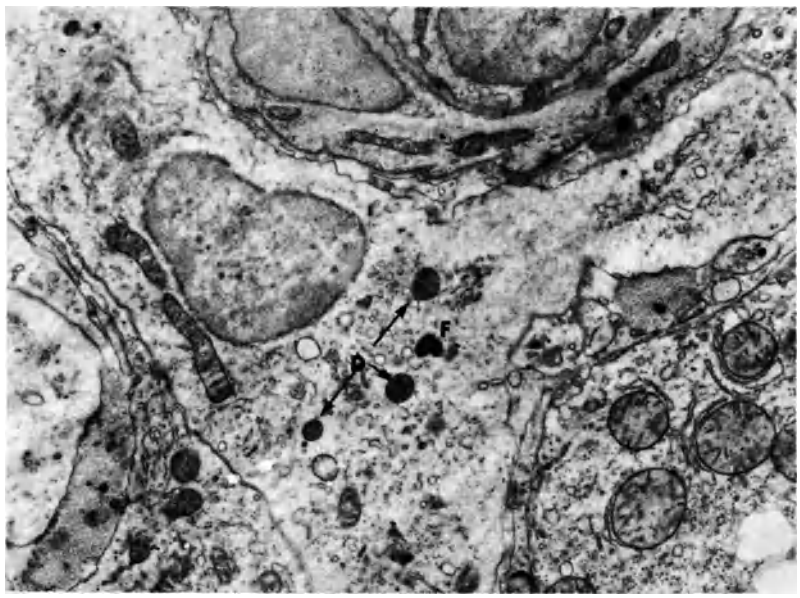

Fig.2. Liver of mouse treated with B.663 (50 mg/kg/day) for 7 days. Dense inclusion bodies (D) with two showing fusion (F) are visible in a Kupffer cell.

With further B.663 treatment the cytoplasmic inclusion bodies increase in number and in diversity of size, shape and electron density. Within some bodies (phagolysosomes) electron-transparent areas of very early crystallisation may be seen (Fig.3). Eventually larger crystals are formed (Fig.4).

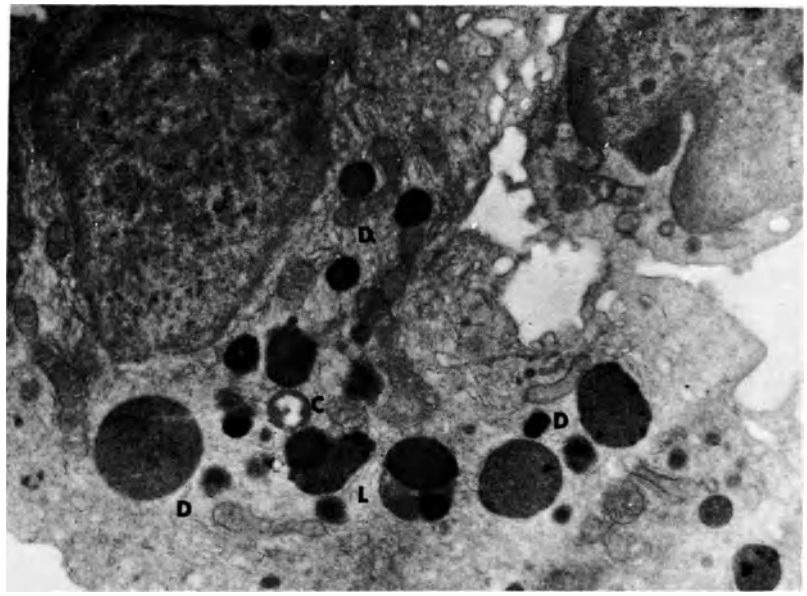

Fig.3 Mesenteric lymph node of mouse treated with B.663 (50 mg/kg/day) for 14 days. Macrophage showing dense bodies (D) of various shapes, sizes and electron densities Phagolysosomes (L) are present and near the center of the field there is early crystal formation within a phagolysosome (C).

Electrophoretic studies of blood serum of orally treated mice have shown binding of B.663 to the lipoproteins of the α and β globulin fractions. These lipoprotein-B.663 complexes are then taken up by the macrophages to form the electron-dense phagosomal bodies. Following fusion with lysosomes, it would appear that the lipoprotein carrier is split off enzymically from the complex, with the resultant crystallisation of the freed B.663.

AVAILABILITY OF INTRACELLULAR B.663

All the evidence - chemoprophylactic activity in

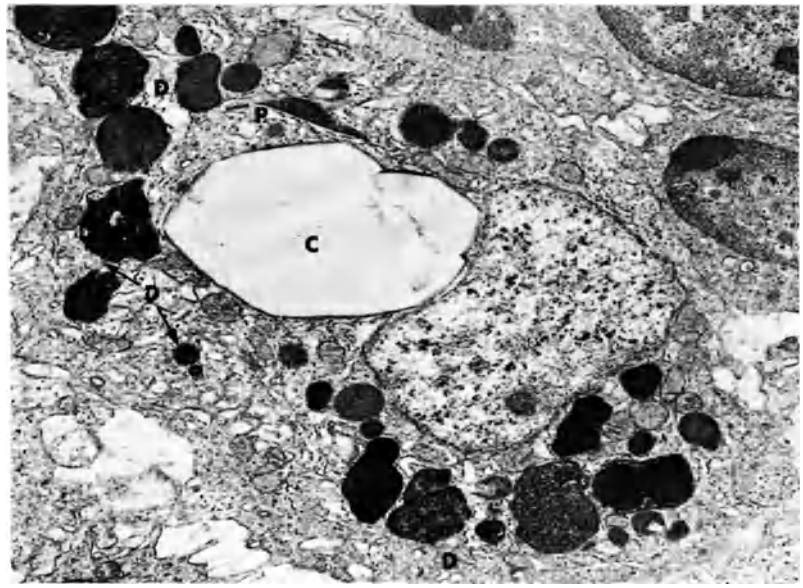

Fig.4. Mesenteric lymph node of mouse treated with B.663 (50 mg/kg/day) for 14 days. Macrophage showing the outline or "ghost" of a fully formed B.663 crystal (C). The compound itself has dissolved out during the fixation process. An earlier "pre-crystal" stage (P) and dense bodies (D) are also to be seen.

tuberculosis of mice (6,11) and hamsters (13), M.lepraemurium infection in mice (14) (all intracellular infections) and particularly the chemotherapeutic response in leprosy of humans - points to the availability of the intracellular drug for the intracellular bacilli. This is unlike the situation which has been observed by du Buy (15) in the case of Brucella infection where intracellular bacilli are sheltered from likewise intracellular tetracycline.

EFFECT ON PHAGOCYTOSIS

The fact that B.663 is concentrated within phagocytes to the extent that crystals develop is evidence of the small extent to which B.663 interferes with the working of these cells and the fact that these crystal-laden cells will phagocytose tubercle bacilli and carbon particles was reported some years ago (8).

Recently, however, we have carried out detailed quantitative investigations of the influence of B.663 on phagocytosis using the classical carbon clearance method of Biozzi, Benacerraf and Halpern (16). Our findings for mice treated via the diet with B.663 at the rate of 50 mg/kg bodyweight daily, are set out graphically in Figs.5-8.

It will be seen (Fig.5) that over the 29 weeks of treatment while there was a progressive fall in the K value for the controls due to ageing (17), there was a very much greater fall in the K value for the treated mice, the difference becoming significant at the 0.001

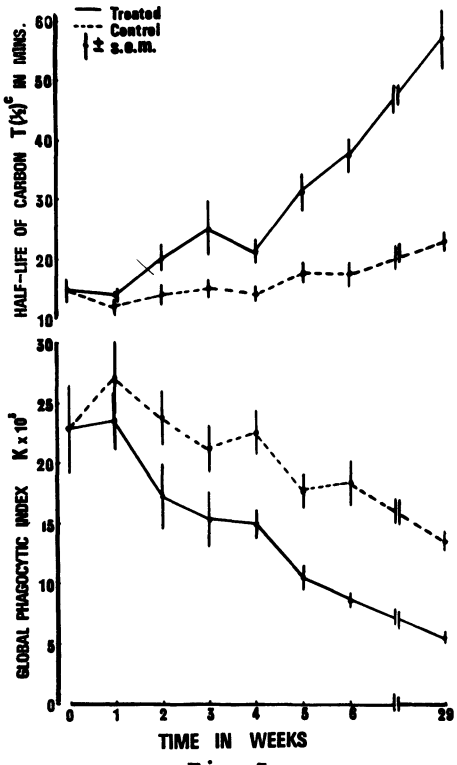

Fig.5

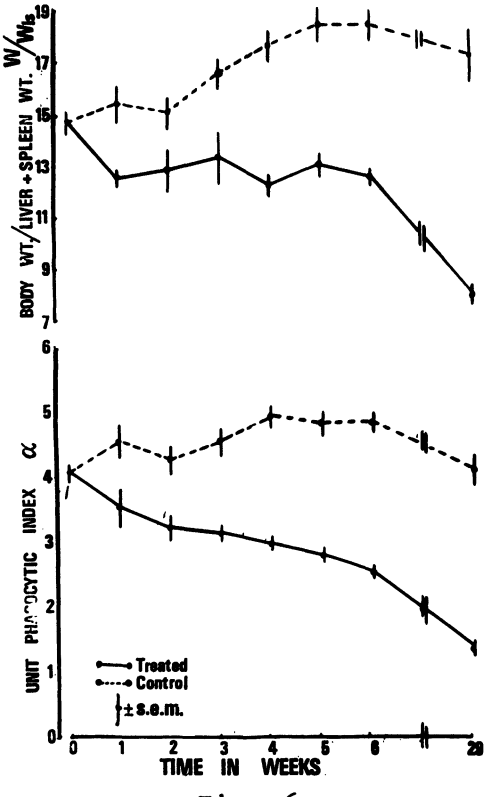

Fig. 6

Fig.5 K and $T(\frac{1}{2})^c$ indices of carbon clearance in mice during a 29 week course of B.663. Vertical line through each point denotes the limits of one standard error of that mean.

Fig.6 Unit phagocytic index α in the same mice.

level as early as the fifth week of treatment. This is also seen in the $T(\frac{1}{2})^c$ value. The profound depression of phagocytosis is likewise apparent when considered on the unit basis, and at the same high degree of significance, as early as the third week (Fig.6).

We have also examined for recovery in phagocytic activity following its depression by 6 weeks of treatment as above (Fig.7). It is clear that there was no substantial improvement during the first 5 weeks after stopping the administration of B.663 but that by the 11th week the K and $T(\frac{1}{2})^c$ values had returned to control levels, but not so the α value (Fig.8).

That there should be reduced phagocytic activity in

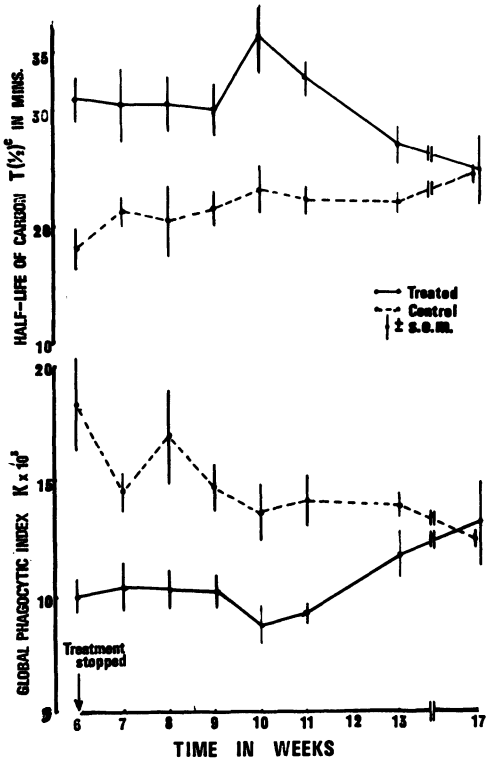

Fig.7

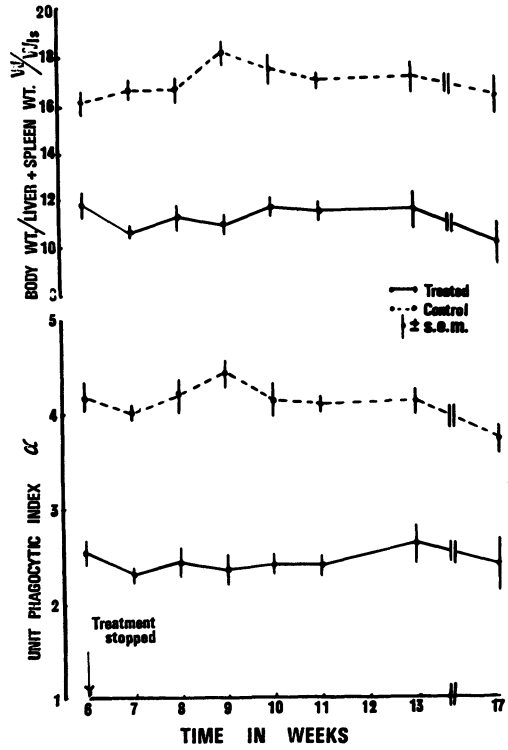

Fig.8

Fig.7 K and $T(\frac{1}{2})^c$ indices of mice following completion of a six weeks course of B.663.

Fig.8 Unit phagocytic index α in the same mice.

B.663-"loaded" animals is not unexpected in view of the obvious curtailment of intra-macrophage space by the sequestered drug. However, the possibility of a direct drug effect on phagocytic function cannot be ruled out.

IMMUNOGOLICAL ASPECTS

Naturally the findings of depressed phagocytic activity have intrigued us, particularly in view of the protective effect of B.663 against erythema nodosum leprosum (ENL) reactions in leprosy patients (18-21). These clinical findings suggest that antigens responsible for the ENL reaction belong to that group of antigens which require to be processed by macrophages in order to stimulate antigen-sensitive thymic-dependent lymphocytes to produce antibodies (22) and that the B.663 depression of phagocytosis reduces the entry of antigen for this processing. It appears feasible also that diversion of lysosomes to deal with phagosomal B.663 may be important in reducing the processing of such antigen as does enter the cells. Bearing in mind too, the effect on lysosomal activity of the anti-mycobacterial polyoxyethylene derivatives of tertiary octyl phenols (23), compounds which also have chemoprophylactic properties, the possibility of a direct effect of B.663 on the lysosomes cannot be discounted. Arising from all of this, the likelihood that B.663 might be of use in autoimmune diseases of the organ-specific type must also be considered.

Recently, antilymphocytic serum (A.L.S.) administered intravenously in mice has been shown by Sheagren and his co-workers (24) to produce what they describe as a "profound" impairment of carbon clearance in mice, most marked at 48 hours where the half-time for clearance was approximately doubled. These workers went on to speculate whether the dose of concomitantly administered drugs cleared by the RES might require reduction or if the blocking activity might help to explain potentiation of immunosuppressive drugs by ALS. Yet this blocking effect looks much less profound when compared with that which we have shown may be obtained with B.663. These very same points are, therefore, possibly even more relevant in the case of B.663.

ACKNOWLEDGEMENTS

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College, Dublin for the electron microscope facilities. Financial support was given by May & Baker Ltd., Dagenham, England, Irish Hospitals Trust Ltd. and Arthur Guinness,Son & Co.(Dublin) Ltd., which is acknowledged with thanks.

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RETICULO-ENDOTHELIAL ACTIVITY AND SOME METABOLIC CHANGES RELATED TO TRAUMA*

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Extensive earlier studies have focused on the metabolic changes after different kinds of trauma. The protein catabolism (2, 3, 4) in such conditions as well as hematological changes (7, 12) are well known but the cause of these metabolic changes is not yet known. Postoperative blood loss or hemolysis can sometimes be responsible for the anemia but does not seem to be the sole cause. It has been proposed that liberation of corticosteroids as a result of "stress" may be responsible for some metabolic changes (19). Finally, some authors believe that substances liberated from the wounded area cause some of the changes. The present aim was to study the possible relationship between the reticulo-endothelial (R.E.) activity after trauma and metabolic changes such as the plasma elimination of folic acid and radioiron.

MATERIAL

Twenty-nine patients divided in 3 groups were studied. Experimental studies were done on 2 dogs and 1 goat.

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* Studies in secondary anemia (V). Supported by the Swedish Medical Research Council and the Swedish Medical Defence Committee.

** International Atomic Energy Agency and Stockholm Cancer Society Fellow on leave from the Institute of Medicine, Bucarest, Romania.

Table 1. Description of patients subjected to surgery

Initial	Sex	Age	General condition at time of op.	Hb g/100ml b.o. x)	ESR * mm/hour b.o.	Operation
H.C.	M	32	good	15,9	10	Extirpation of ruptured meniscus
G.B.	M	25	good	14,9	1	Repair of ruptured ant.cruc. lig.
G.K.	M	27	good	14,0	1	Repair of ruptured ant.cruc. lig.
A.L.	M	21	good	16,0	2	Repair of ruptured ant.cruc. lig.
H.K.	M	21	good	16,6	1	Extirpation of corp.libr.gen.
O.E.	F	52	good	11,8	19	Nailing of fract. radii et ulnae (Accident one week before operation)
L.W.	M	52	Severe burns (50-60 %)	9,0	72	Amputation of both legs
F.M.	F	82	good	11,0	29	Nailing of fract.colli fem. (Accident one week before operation)

x) b.o. = before operation

* ESR = Erythrocyte sedimentation rate.

Effect of Surgical Trauma

Eight patients aged between 20 and 82 years (mean age 38) were studied before and after surgical operations (see table 1). Of these, 5 patients (aged 20-25 years) were submitted to meniscus operations or suture of ruptured

ligaments of the knee joint. The operations were done in a blood-less field and no signs of postoperative hematomas were seen. Two elderly patients were operated for fractures and one (52 years old) had a leg amputation after an electrical burn. All these patients were investigated before and 24 - 48 hours after operation. In 2 dogs a tissue injury was simulated by the injection of autologous muscular extract.

Effect of Steroids

Six patients with third degree burns, covering from 10 to 30 per cent of the body surface, were studied prior to and after treatment with 40 mg Prednisolone daily for 3 days. These patients were aged between 35 and 56 years with a mean age of 50 (see table 2). The study took place 3 - 4 weeks after the accident. To compare the values of the burned group with normals eight healthy volunteers aged between 50 and 78 years (mean age 67) were studied.

Table 2. Description of patients with burn injuries

Initial	Sex	Age (years)	Burns, percentage of body surface (per cent)	Other observations	Hb (g/100 ml) b.c.[x]
B.A.	M	51	30	Diabetes mellitus	10,0
M.M.	M	35	15	-	12,2
H.E.	M	52	15	-	13,8
M.D.	M	56	20	-	8,2
L.G.	F	56	15	Reumatoid arthritis. ACTH-treatment when burned.	10,5
D.I.	M	49	10	-	12,8

x) b.c. = before corticosteroids

Effect of Vaccination

To evaluate the possible effect of antigenic stimulation on the RE-system, 7 healthy patients (aged 23-47 years, mean age 34) were given 7 mg of radio-gold before and after anti-variolic vaccination. These patients were all subjected to revaccination and they all had very slight local reactions. One goat previously sensitized to D-antigen and given a new antigen dose in order to produce D-antibody was also studied.

METHODS

Each study consisted of the simultaneous intravenous administration of four substances. Colloidal ^{198}Au was given in an amount of 0,05 - 0,1 mg and an activity 10 μCi. It had a mean particle size of 25 mμ. ^{125}I-Rose Bengal was administered in the amount of 12 μCi and ^{59}Fe as ferric citrate in a dose around 0,15 - 0,30 μg and an activity of 5 μCi. The folic acid was injected in a dose of 15 μg per kg body weight. The vaccinated patients got only folate and 7 mg of colloidal gold corresponding to 10 μCi. All the patients were fasting over night before the study.

Blood samples were obtained before the injection, and at 3, 7, 11, 15, 20, 30, 40, 50, 60 and sometimes 90 and 120 minutes after the injection. The radioactivity in the whole blood (3 ml) was measured in a Packard Tricarb Automatic counter with a double channel gamma spectrometer. The radio-activity of each isotope was measured in the following channels: 22-46 Kev for ^{125}I, 370-450 Kev for ^{198}Au and 900-1700 Kev for ^{59}Fe. The following correction equations were used to calculate the Compton effect of ^{59}Fe on ^{198}Au and ^{125}I, and of ^{198}Au on ^{125}I. Summation effects were assumed to be negligible.

$$^{198}Au = {}^{198}Au\text{-channel} - 0,25\ {}^{59}Fe\text{-channel}.$$

$$^{125}I = {}^{125}I\text{-channel} - 0,15\ Au\text{-channel} - 0,07\ {}^{59}Fe\text{-channel}.$$

The Compton correction factors (0,25, 0,15 and 0,07) were measured in the same well type NaI crystal as all samples. The folic acid assay was performed in separate plasma samples taken before and at 3, 15, and 30 minutes after the injection. The serum folate activity was bioassayed with L.casei as earlier described (16). Hemoglobin (Hb) concentrations and erythrocyte sedimentation rates (ESR) were examined by routine methods. The final data were calculated by hand and by computer.

The anaesthetized dogs received an injection of colloidal gold (4 μCi ^{198}Au) and iron (2 μCi ^{59}Fe) before and after the following experiment. A piece of thigh muscle was removed, crushed in physiological NaCl, centrifuged, homogenized in a Potter-Elvehjem glass homogenizer, and stored frozen for 10 days. During this time the wound healed. After this interval the anaesthetized dogs were given 10 ml of the solution containing the muscle extract intravenously. Two hours later the radio-active materials were again given, and blood samples taken at the same time intervals as in man.

A goat used for studies of anti-D-gamma globulin and immunized with D-antigen several months prior to the present study, was examined by means of colloidal radiogold and radioiron in about the same doses as used in man. The goat was studied before and 24 hours after injection of a new antigen dose.

Rates of plasma elimination are expressed as 'k' (the coefficient of regression of the ln of activity on time) and for ^{125}I Rose Bengal called RB_k, for ^{198}Au, Au_k, for ^{59}Fe, Fe_k, and for folic acid it was called FA_k. For gold and Rose Bengal two different slopes were often observed. The second slope (Au_{kII}, RB_{kII}), extrapolated to zero time, was subtracted from the experimental values of the first slope to obtain a corrected first slope (Au_{kI}, RB_{kI}) as described earlier (14). For iron and folate only one slope was observed.

RESULTS

Effect of Trauma

Table 3 shows the different parameters before and after operation. It can be seen that statistically highly significant increases in the elimination rates occur for gold, iron, and the second phase of Rose Bengal, whereas for other parameters the changes are not statistically significant. It is thus suggested that an increase in RE-uptake is caused by surgical trauma. The same thing also seems to happen after burns, as the Au_{kI} in this group is significantly higher than the Au_{kI} in the group of elderly healthy patients. The burned group also had a significantly higher elimination rate of radioiron than the control group (table 4). On the other hand, the effect of trauma could not be confirmed in the simulated trauma experiment. The injection of autologous muscle extract did not significantly affect the elimination rate of radiogold or radioiron.

Table 3. Effects of operation on the iron and folate metabolism and on RE-function

Parameters	No of subjects	Before operation M ± SE	No of subjects	After operation M ± SE	Significance of difference between pre- and postoperative values
"RE-function" (Au_{kI})	8	0,21±0,022	8	0,28±0,027	0,001<p<0,01
"RE-function" (Au_{kII})	4	0,042±0,012	3	0,013±0,003	n.s.[x]
Iron elimination (Fe_k)	7	0,011±0,003	7	0,024±0,003	p<0,001
Folate elimination ($F.A_k$)	8	0,08±0,015	7	0,066±0,012	n.s.
Folate concentration prior to injection	5	4,93±1,19	5	4,33 ±0,96	n.s.
Folate concentration 15' after injection ($FA_{15'}$)	5	133,0±46,3	5	78,0±13,5	n.s.
Rose Bengal elimination (RB_{kI})	8	0,12±0,007	8	0,13±0,008	n.s.
Rose Bengal elimination (RB_{kII})	8	0,010±0,0015	8	0,016±0,002	p<0,001

[x] n.s. = not significant

Table 4. Effect of burns on the iron metabolism and on RE-function.

Parameters	No of subjects	Controls M ± SE	No of subjects	Burns M ± SE	Significance of difference between the groups
"RE-function" (Au_{kI})	8	0,16±0,018	6	0,29±0,04	$p < 0,001$
"RE-function" (Au_{kII})	2	0,037±0,026	5	0,07±0,02	$p > 0,05$
Iron elimination (Fe_k)	8	0,007±0,0005	6	0,020±0,003	$p < 0,001$

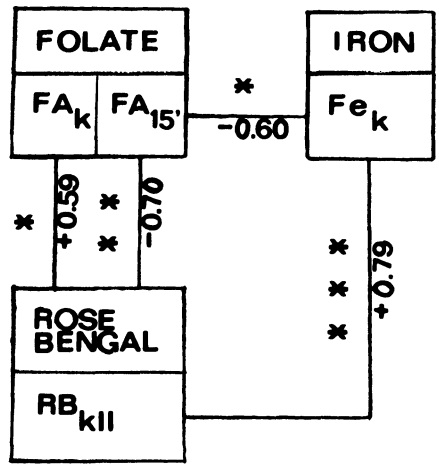

Fig. 1. The lines indicate statistically significant correlations, signs show the direction of the correlation. x = $0,01 < p < 0,05$, xx = $0,001 < p < 0,01$, and xxx = $p < 0,001$. It is seen that the more rapidly iron is eliminated, the steeper is the second slope of Rose Bengal and the lower is the folic acid activity at 15 minutes after injection. The more rapidly folic acid is eliminated the steeper is the second slope of Rose Bengal.

Table 5. RE-activity in burned patients before and after steroid treatment. (M ± SE)

Parameters	No of subjects	Before steroids	No of subjects	After steroids	Significance of difference before and after steroid treatment
"RE-function" (Au_{kI})	6	0,29±0,04	6	0,27±0,04	n.s.[x]
"RE-function" (Au_{kII})	5	0,07±0,02	4	0,07±0,02	n.s.
Iron elimination (Fe_k)	6	0,020±0,003	6	0,012±0,002	$p < 0,001$
Rose Bengal elimination (RB_{kI})	2	0,14 and 0,09	2	0,13 and 0,11	n.s.
Rose Bengal elimination (RB_{kII})	2	0,01 and 0,0008	2	0,01 and 0,01	n.s.

x) n.s. = not significant

The correlation between folate, iron, and the second slope of the Rose Bengal curves are illustrated in fig. 1. It is seen that the more rapidly iron is eliminated, the steeper is the second phase of Rose Bengal elimination. The more rapidly the folate is eliminated, the more rapidly does the second slope of Rose Bengal (RB_{kII}) fall.

Effects of Steroids

Corticosteroids could not be demonstrated to inhibit significantly the elimination rate of radiogold but they did significantly depress the elimination rate of radioiron (table 5).

Effect of Immunization

In 7 patients submitted to antivariolic revaccination the elimination rate of radiogold was not changed after vaccination (table 6). Nor did it change after D-immunization (table 7)

Table 6. Effect of vaccination on "RE-function" and other parameters

Parameters	Before vaccination M ± SE	After vaccination M ± SE	Significance between values before and after vaccination
"RE-function" (Au_{kI})	0,092±0,008	0,092±0,009	n.s.[x]
Folic acid elimination (FA_k)	0,13±0,011	0,10±0,013	n.s.
Erythrocyte sedimentation rate in mm/hour (ESR)	3,7 ± 0,55	3,6 ± 0,81	n.s.
Serum-iron conc.in mg/ 100 ml (Se-Fe)	0,120±0,082	0,082±0,099	$0,001 < p < 0,01$
C-vit.conc. in mg%	1,39 ± 0,11	1,30 ± 0,12	n.s.

x) n.s. = not significant

Table 7. Animal experiments

Animal	Clearance	Before injection[x]	After injection
Dog I	Au_{kI}	0,315	0,198
	Au_{kII}	0,046	0,007
	Fe_k	0,007	0,010
Dog II	Au_{kI}	0,345	0,30
	Au_{kII}	0,022	-
	Fe_k	0,010	0,007
Goat	Au_{kI}	0,20	0,16
	Au_{kII}	-	-
	Fe_k	0,0064	0,0059

x) in dogs: autologous muscle extract
in goat: D-antigen

as studied in a goat. In accordance with this, no significant increase in erythrocyte sedimentation rate was found, nor did the serum ascorbate decrease in the vaccinated patients. On the other hand, the serum iron decreased significantly ($0,001 < p < 0,01$) after vaccination.

DISCUSSION

Discussion of the Methods

To study the rate of blood clearance of particles by the RE-cells, here called "RE-activity", albumin aggregates, inactivated erythrocytes, lipid emulsions, and colloids in various doses have been used (5, 6, 8, 11, 20) and, in part, the results depend on the methods used. Radiogold was selected as a test substance because it corresponds to the general principles established by Benacerraf (1) and was commercially available for clinical studies and simple to use. The present form and doses of colloidal gold were used tentatively, and studies are in progress to see if different parts of the RE-system can be distinguished by using different methods, or if different doses can distinguish effects on circulation from effects on phagocytosis.

The present study is insufficient to answer the question if the effects observed are caused by changes in phagocytic activity or circulation. However, circulatory changes would have been expected to affect the first phase of Rose Bengal elimination (18). No such effect was noted.

Discussion of the Results

Possible increase of RE-activity. In the present study a rapid colloidal gold elimination rate, a rapid elimination rate of iron and also a rapid elimination of (the second phase) Rose Bengal are found after a trauma. The increase in elimination was significant as compared to the control group. Similarly, the folate elimination was numerically but not significantly increased. It is known that about 90 per cent of the colloidal gold particles are taken up by the reticulo-endothelial cells, especially by the Kupffer cells of the liver (18, 19). It is also known that iron can sometimes be demonstrated with histochemical methods in RE-cells. It is thus possible that the rapid elimination of iron and gold indicate a high RE-activity.

However, no significant correlation was found between the iron- and the gold-elimination. Moreover, after vaccination, the serum iron fell although the gold-elimination did not increase and steroids normalized the iron elimination but not that of gold. Therefore, there are probably differences between the effect on iron and on colloidal gold. It is not known if these differences are caused by different RE-cells, differences in receptor sites or differences unrelated to RE-cells.

In earlier studies of postoperative patients, which also demonstrated high elimination rates of iron (12) and albumin (2, 3, 4, 16), a correlation was observed between increased hemolysis and the increased elimination rate of iron. Conceivably an increased RE-activity could lead to erythro-phagocytosis and hemolysis as well as to the disappearances of iron. It is not known, however, if increased RE-activity causes both hemolysis and rapid iron elimination, or if it is the hemolysis which affects iron metabolism (10, 12). The latter possibility is considered to be less likely, since the increased iron elimination appears as early as 24 hours after a trauma (10).

An increased uptake in RE-cells could be caused either by an increased blood flow past the phagocytes, or by an increased phagocytic activity in the cells. The Rose Bengal elimination (RB_{kI}), generally considered to increase when the liver circulation is increased (18), was unchanged in our cases. It is thus not believed that circulatory changes alone could have caused the changes found. The traumata in the present group were small and unlikely to cause circulatory changes lasting for 24 hours. In similar cases we have earlier shown that no circulatory changes will take place (13).

The second slope of Rose Bengal (RB_{kII}) was found to be steeper after a surgical trauma. Generally this slope is believed to depend on factors other than the hepatic cell function. Seventy per cent of Rose Bengal are bound to albumin and the rest to different globulin fractions (15, 18). It is conceivable that the same carrier deals with the transport of fractions responsible for the second slope of Rose Bengal, with the transport of iron, and with some part of the folic acid activity. The increased elimination rate could be due to a more rapid elimination of related carrier substances. Naturally, alternative explanations are possible.

Possible pathogenesis of high RE-activity. An increased phagocytosis of the colloidal particles, proteins and substances carried by them could be regarded as a link in a

normal defence system. Possibly red cells could be phagocytosed as a side effect. However, the question remains how this system is activated. Present experiments were insufficient to demonstrate an immunological alarm in vaccinated patients and in our experiment in a goat.

Liberated proteins after cell damage ("wound hormones") have been discussed, but not isolated. In our studies in 2 dogs the muscle extract injected gave no significant increased RE-activity. The few present animal studies neither support nor refute the existence of such substances.

Conceivably, elimination of colloidal particles can be explained by hyperplasia of RE-cells, as described in some chronic diseases (10). In the cases studied here the interval between the trauma and the RE-function test was so short that hyperplasia seems unlikely to have occurred. Nor is it likely that leakage of albumin (5) gold or transferrin into the traumatized area would explain the present results, since the surgical trauma was minor. However, further studies are required to explain the results reported.

The results found in patients after surgery resemble those in groups of patients with cancer or inflammation (9) which suggests a non-specific effect on the RES. The enhanced RE-activity after surgical trauma could be a part of general defence mechanisms.

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THE BEHAVIOUR OF MACROPHAGES IN LUNG CULTURES AFTER EXPOSURE TO CIGARETTE SMOKE. EVIDENCE FOR SELECTIVE INHIBITION BY PARTICULATE MATTER AND STIMULATION BY THE GAS PHASE OF CELL METABOLISM OF ALVEOLAR MACROPHAGES

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In contrast to the extensive experimental work on the biological activity of cigarette smoke condensates or extracts, there are few experimental investigations concerned with the biological effects of fresh native cigarette smoke itself on animals, tissues or cells. Data obtained with fresh cigarette smoke would appear to be more directly related to the important problem of the role of cigarette smoking in the genesis of pathologic changes in humans than data obtained with cigarette smoke condensates or extracts. For a number of years, therefore, we have used two types of model systems :

1. Inhalation studies in mice with fresh cigarette smoke (1,2).
2. Exposure of mouse cell, tissue and organ cultures to puffs of fresh cigarette smoke (3,4,5).

While studies on living animals, such as mice, are especially suitable for assessing long range and late effects of chronic inhalation of cigarette smoke, additional studies on cultures are necessary in order to determine early and sequential effects of cigarette smoke on the cell and its metabolism.

The present report deals with biological effects of charcoal-filtered whole cigarette smoke or its gas phase on alveolar macrophages and epithelioid cells in cultures of lung explants from Snell's mice and Golden hamsters. Charcoal-filtered cigarette smoke was utilized because we had previously observed in a variety of types of

cultures and in different types of cells, that unfiltered whole cigarette smoke, or its gas phase alone, evoked striking cell damage. This was characterized by a sequence of regressive changes, namely inhibition of nucleoprotein metabolism, loss of RNA and loss of DNA, resulting in pycnosis and cell death. Such "cytotoxic" effects were, however, absent after the same cigarette smoke passed through activated charcoal, which adsorbs important cytotoxic factors, such as acrolein (3, 4). Furthermore, exposure of 3T3 cells to the gas phase from charcoal-filtered cigarette smoke resulted in an enhancement of growth, increased mitotic activity, DNA synthesis and cellular atypism (5). An exploration of the biological effects of this "nontoxic" charcoal-filtered cigarette smoke on growth and DNA metabolism of alveolar macrophages seemed of special interest, not only because of their significance for clearing processes of the respiratory tract, but also because it has been reported that unfiltered cigarette smoke has a depressant effect on the *in vitro* antibacterial activity of rabbit alveolar macrophages (6). Although there is an excellent technique (Myrvik, 7,8) for obtaining cultures of rabbit alveolar macrophages with a high degree of purity by flushing them out of the lung, we used for our studies cultures of alveolar macrophages grown out *in situ* from lung explants. This type of culture has the advantage that there is not only the lung explant itself, but also a monolayer outgrowth of alveolar macrophages, epithelioid and fibroblastic cells (Fig.1). Thus the alveolar macrophages can be studied in a cellular environment which resembles more closely that in the intact animal. Furthermore, this system appeared to be especially suited to the assessment of the effects of cigarette smoke, since sequential alterations can be followed in living cultures by phase microscopy as well as in fixed preparations at the single cell level by the use of correlated cytological and cytochemical methods, such as microspectrography (9).

Lung explant cultures from Snell's mice and Golden hamsters were prepared on coverslips in Falcon dishes and grown in EagleDulbecco medium with 20% calf serum at 37°C in a CO_2 incubator as described previously (5). Before exposure to cigarette smoke, 6-7-day old lung explant cultures were matched under the phase microscope, not only in regard to size of lung explant, but also in regard to number of alveolar macrophages and epithelioid cells. The matching of such living cultures is difficult and requires an experienced cytologist. In each experiment a minimum of 6 groups was used, each group consisting of 4 matched

cultures: 2 controls, one exposed to whole charcoal-filtered (WCF) cigarette smoke and one exposed to the gas phase (GCF) of charcoal-filtered cigarette smoke. Altogether, over 20 different experiments, each comprising a minimum of 24 cultures on coverslips, were carried out for the present study. In order to expose the cultures to puffs of fresh cigarette smoke, a CSM 12 Filtrona Smoking Machine, to which plastic compartments were attached (see Figs. 2a, 2b), was utilized as previously described (5). For the production of the gas phase the cigarettes were placed in front of a special filter holder containing a Cambridge Filter Disc N° 9-86, Phipps and Bird, Richmond Virginia. The charcoal-filtered cigarette used was the same as that utilized in our previous studies (3,4,5). It is important to mention that we found it necessary to store cigarettes with freshly prepared activated charcoal in plastic bags at 5°C; otherwise a marked decrease in the adsorbing activity of charcoal for cytotoxic factors from the gas phase of cigarette smoke was observed within 2 months. The activated charcoal in the cigarette filters was prepared from Coconut. Filters contained 75 mg per filter, mesh size was 12 x 30, and Iodine Index was 140.

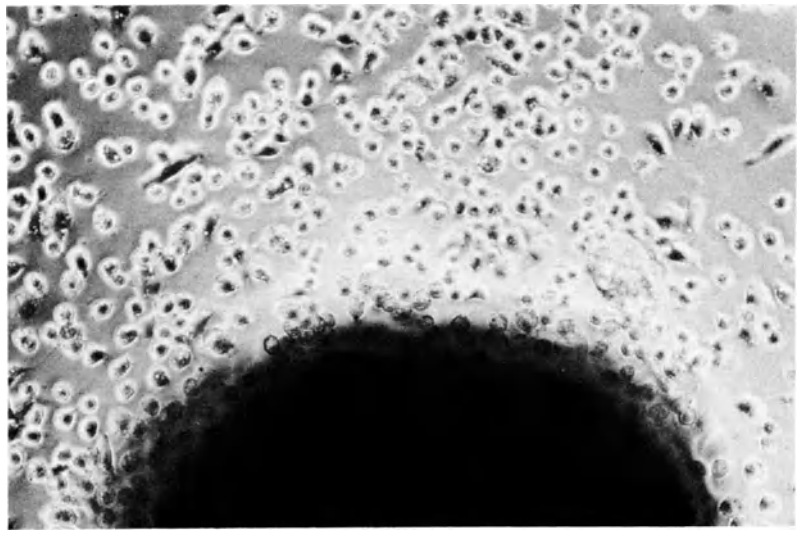

Fig. 1: Photomicrograph (phase microscope) of a living culture of a 6-day old lung explant from Snell's mouse. Note lung explant in lower part, surrounded by outgrowing alveolar macrophages and epithelioid (fibroblastic?) cells. x 160.

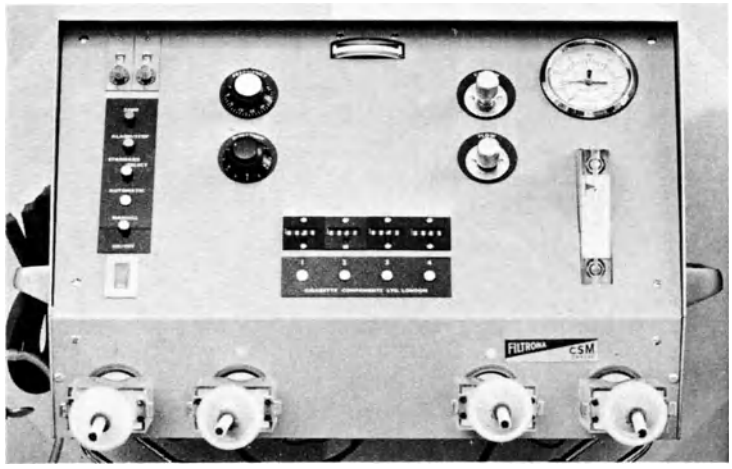

Fig. 2a: Photograph of Filtrona Smoking Machine CSM 12 for exposure of culture to puffs of fresh cigarette smoke.

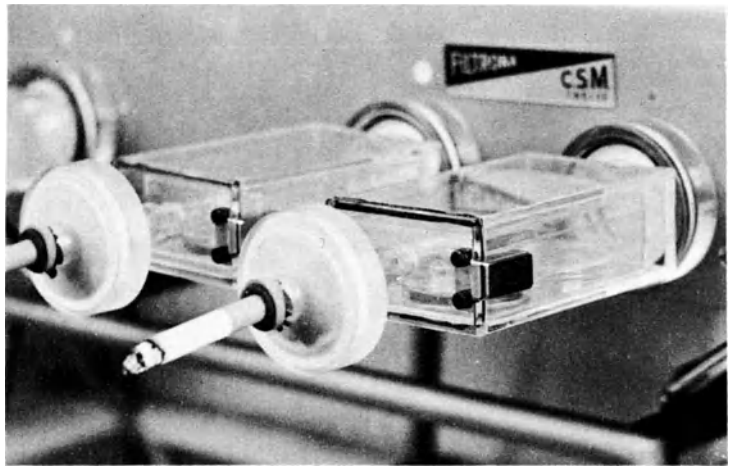

Fig. 2b: Side view of plastic compartments of Filtrona CSM 12 for placing cultures. Note also attachment for Cambridge Filter holder.

Each culture was exposed daily to 2 - 4 puffs. The volume of each puff was 8 ml, duration was 2 seconds, and interval between each puff was 58 seconds. After each exposure the medium was changed immediately and the cultures were returned to the CO_2 incubator at 37°C. All living cultures were examined regularly under the phase microscope. For cytological and cytochemical examinations, cultures were fixed and stained as previously described (3,4). For the study of DNA synthesis, Feulgen microspectrography (9), and microfluorometry (5) were utilized. In addition, DNA synthesis was studied by tritiated thymidine labeling in radioautographs. For this purpose the cigarette smoke treated and control cultures were exposed for 30 minutes to 1.5 ml of medium containing 0.75 µC tritiated thymidine, and the radioautographs prepared according to Feinendegen (10). The relationship between numbers of macrophages and epithelioid (fibroblastic) cells was evaluated under the phase microscope on living cultures as well as in fixed and stained preparations. For the counting of numbers of outgrowing cells, all cells present in a monolayer on the coverslips were counted. From these data the ratio between epithelioid cells and alveolar macrophages was obtained.

There were three essential features of the alveolar macrophages in control lung explant cultures derived from either Snell's mice or Golden hamsters.

1. The number of outgrowing alveolar macrophages was always larger than the number of outgrowing epithelioid or fibroblastic cells (Fig. 3), giving a mean ratio between epithelioid cells and macrophages of approximately 0.6 (Table 1).
2. Alveolar macrophages showed mitotic activity (Fig.4).
3. Alveolar macrophages manifested DNA synthesis, as demonstrated by incorporation of tritiated thymidine (Fig. 5 and Table 1) and by Feulgen microspectrography (Fig. 6). The data indicate that the DNA content is variable in control alveolar macrophages, many of the cells containing more than the basic value of 2 DNA (1.5 arbitrary units).

The salient features observed in experimental cultures of outgrowing macrophages and epithelioid cells after exposure of lung explants to either WCF cigarette smoke or to GCF alone, are presented in Table 1. The results were reproducible throughout the entire experimental series.

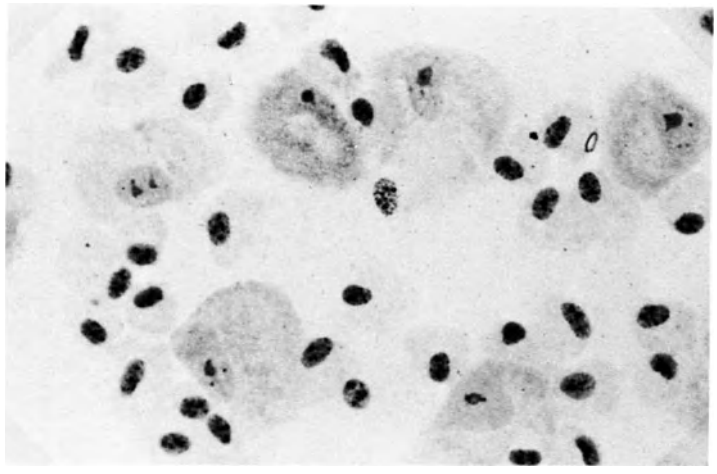

Fig. 3: photomicrograph of outgrowing cells from a 15-day old control lung explant from Snell's mouse. Note preponderance of macrophages and some epithelioid (fibroblastic ?) cells. There is labeling by 3H TdR of some of the macrophages. Giemsa stain of radioautograph. x 375.

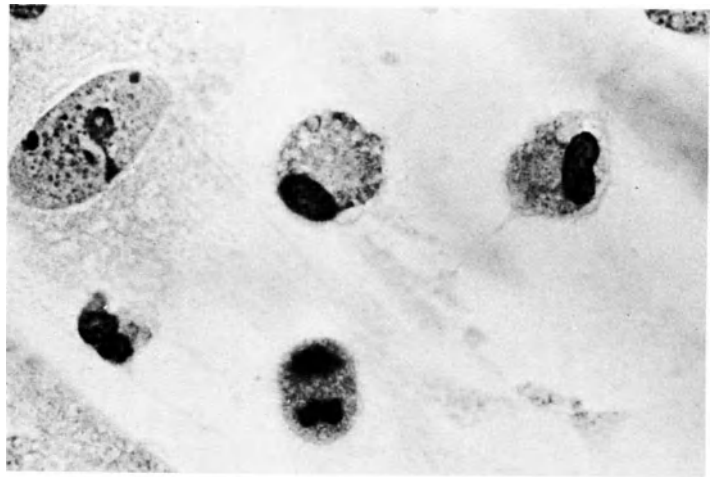

Fig. 4: Photomicrograph of outgrowing macrophages from a 20-day old control explant from Golden hamster, showing mitosis of macrophages. H. & E. x 938.

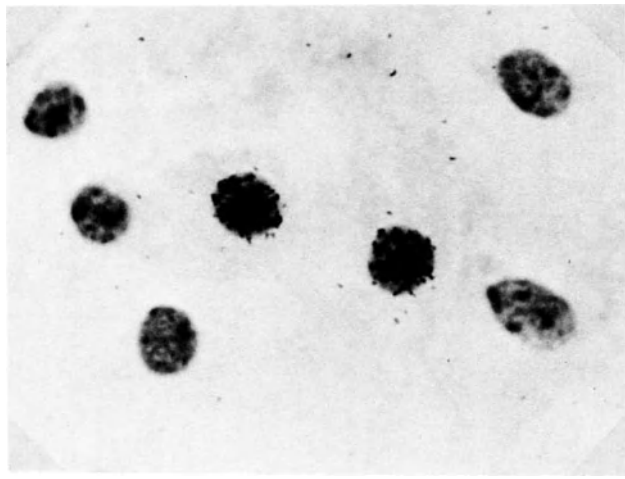

Fig. 5: Photomicrograph of outgrowing macrophages from a 20-day old control lung explant from Golden hamster, showing incorporation of tritiated thymidine. Giemsa stain of radioautograph. x 938.

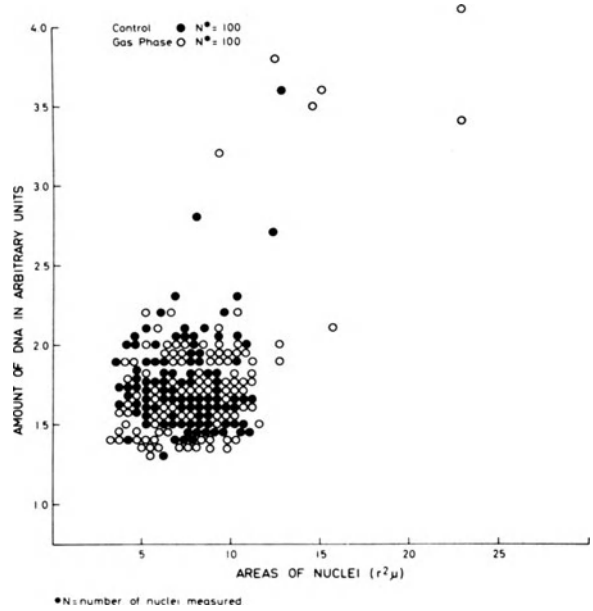

Fig. 6: Relationship between DNA content (Feulgen microspectrography) and nuclear size of alveolar macrophages from 21-day old control lung explants and explants of the same age exposed to 14 x 2 puffs (8ml puff volume) of the gas phase from charcoal-filtered fresh cigarette smoke. Note variability of DNA content in control macrophages and higher DNA values and larger nuclei after exposure to the gas phase.

Table 1

Frequency and DNA synthesis of epithelioid (fibroblastic ?) cells and alveolar macrophages from lung explants (Snell's mice) in controls and cultures exposed repeatedly (4 - 6 times) to 4 puffs of whole smoke or its gas phase from charcoal-filtered cigarettes.

Type of culture	Average number of cells in cultures*	Mean ratio of frequency epithelioid cells / macrophages	DNA synthesis (^{3}H TdR) epithelioid cells (Frequency of labeled cells)**	DNA synthesis (^{3}H TdR) macrophages (Frequency of labeled cells)**
Control n = 10	2200	0.6 ± .18	2.9 ± .65	2.1 ± .41
Gas Phase (GCF) n = 10	2100	0.7 ± .21	2.8 ± .49	6.4 ± .95 p = 0.005
Control n = 9	2500	0.6 ± .17	2.6 ± .50	2.0 ± .39
Whole Smoke (WCF) n = 9	1100	5.4 ± 1.51 p = .005	2.9 ± .44	5.6 ± 1.1 p = 0.005

n = number of cultures examined * = entire monolayer

** = the minimum number of grains per nucleus to consider a cell as labelled was 10 grains.

As can be seen from Table 1, after exposure to WCF smoke the number of outgrowing cells was markedly reduced, while after exposure to GCF smoke the number was essentially the same as in the control cultures. This reduction of total number of cells following WCF smoke did not involve the epithelioid cells, but was a selective damage to alveolar macrophages by the cigarette smoke. Already after one to two daily exposures of 4 puffs the cytoplasm of many alveolar macrophages showed vacuolization and shrinkage, the nuclei became granulated and pycnotic, and macrophages displayed an increasing tendency to detach from the coverslips. The ratios between number of epithelioid cells and macrophages reflect very well the selective damage to macrophages. Whereas in the controls or GCF exposed cultures there was an epithelioid cell/macrophage ratio of 0.6, that is, there were always more macrophages than epithelioid cells (Fig.3), after exposure to WCF smoke there were always more epithelioid cells than macrophages (Fig.7) resulting in a completely altered and reversed ratio (Table 1). This effect was observed in a number of cultures following only 3 daily exposures to 4 puffs of smoke per day. It thus appears that WCF cigarette smoke selectively damages alveolar macrophages which are proliferating and migrating from mouse or hamster lung explants. In view of the fact that the gas phase alone did not have this effect, it is reasonable to assume that the damage was due to particulate matter in the cigarette smoke.

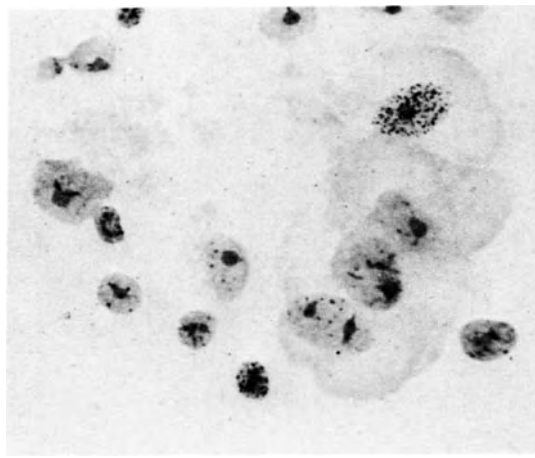

Fig. 7: Photomicrograph of outgrowing cells from a 15-day old lung explant from Snell's mouse, after exposure to 6 x 4 puffs of whole charcoal-filtered cigarette smoke. Note reduction of number of macrophages in relation to number of epithelioid cells, as compared with Fig. 3.

Note also incorporation of tritiated thymidine in remaining macrophages and epithelioid cells. Giemsa stain of radioautograph. x 375.

Although exposure to WCF smoke did not affect directly number or morphology of the epithelioid cells, there was however an indirect effect, in that subsequent to the loss of macrophages, a gradual alteration of the growth behaviour of the epithelioid cells was noted. While in control cultures the majority of epithelioid cells were very well separated, in other words showed contact inhibition (see Fig.8), in cultures previously exposed to WCF smoke the epithelioid cells displayed a loss of this contact inhibition, as well as an increase in size of cells, nuclei, and nucleoli. There was also a progressive tendency to sheet and criss-cross formation, which was particularly striking in older cultures (see Fig.9). Since this altered behaviour of the epithelioid cells in cultures exposed to WCF smoke was always confined to areas depleted of macrophages, it would appear that the presence of macrophages in controls influenced the growth behaviour of these epithelioid cells. It is reasonable to suggest that in these lung explant cultures macrophages were necessary to maintain contact inhibition of epithelioid cells and thus prevent irregular growth. Whether alveolar macrophages play also a role in maintaining orderly growth of bronchial epithelium in the intact host cannot be answered at present, but needs further thorough exploration. In order to probe into this problem, inhalation studies in Snell's mice with the same charcoal-filtered cigarette smoke have been started in our laboratory. Should these *in vivo* studies confirm the *in vitro* results, that is if inhalation of charcoal-filtered cigarette smoke would result in loss of macrophages followed by irregular growth of bronchial epithelium, then the significance of alveolar macrophages would be greatly increased. In other words, such data would indicate that alveolar macrophages are not only important for clearing, but also for growth regulating processes of the respiratory tract.

A selective but different effect on alveolar macrophages was observed when DNA synthesis was studied. This effect, however, was not due to the particulate matter, but to the gas phase of charcoal-filtered cigarette smoke; and instead of inhibition of cell metabolism, the change was one of stimulation of DNA synthesis. As can be seen from Table 1, after exposure to either whole

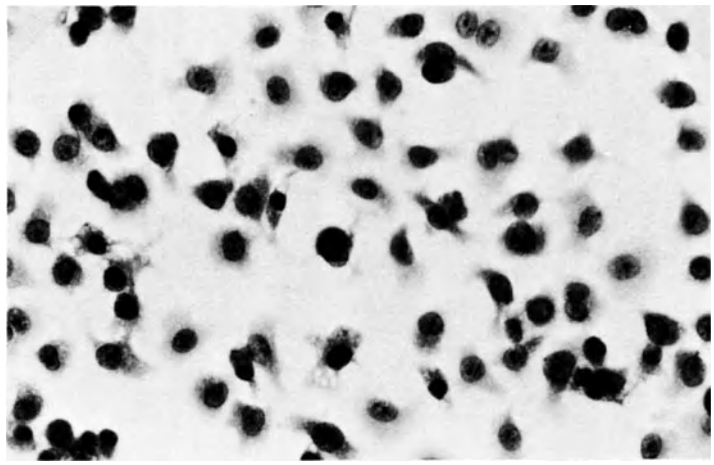

Fig. 8: Photomicrograph of outgrowing cells from a 24-day old control lung explant from Snell's mouse, showing some epithelioid cells (well separated). Note abundance of macrophages. H. & E. x 375.

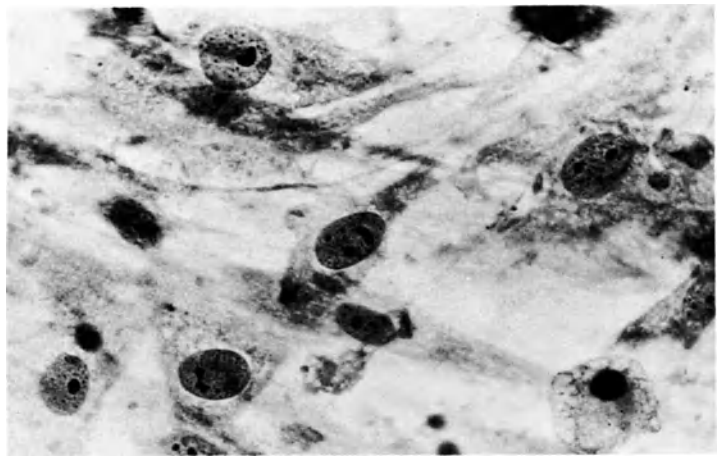

Fig. 9: Photomicrograph of outgrowing cells from a 24-day old lung explant from Snell's mouse, after exposure to 37 puffs (12 exposures) of WCF smoke. In comparison with control (see Fig.8), note larger size of nuclei and nucleoli of epithelioid cells, lack of contact inhibition, sheet formation, and criss-cross of cultures. Note also absence of macrophages. H. & E. x 375.

smoke or to the gas phase epithelioid cells showed no differences in DNA synthesis, compared with controls. In contrast, there was stimulation of DNA synthesis in the alveolar macrophages, following exposure to cigarette smoke (Table 1, Fig. 6). Since the gas phase (GCF) alone produced this effect, it is justifiable to conclude that the stimulation of DNA synthesis in the small number of macrophages surviving after exposure to whole smoke (WCF) was also due to gas components contained in the cigarette smoke.

The observation of a differential effect on alveolar macrophages and epithelioid cells due to particulate matter from charcoal-filtered cigarette smoke, although both types of cells were present within the same monolayer of the culture, deserves consideration. It indicates not only that the activated charcoal did not adsorb all components which inhibit cell metabolism, but also that alveolar macrophages are more sensitive than epithelioid cells to the damaging components. Whether this increased sensivity of alveolar macrophages is related to their highly specialized function as phagocytes must await studies on many more types of cells, including macrophages from other regions. The concept that phagocytic cells may be especially sensitive to cigarette smoke is supported by the work of Eichel and Shahrik, who reported that charcoal-filtered cigarette smoke impaired the function of human oral polymorphnuclear leukocytes (11). Our own studies also suggest that charcoal-filtered cigarette smoke impairs phagocytic function of alveolar macrophages. Extensive search in living cultures as well as in fixed preparations has not revealed engulfed particulate matter of charcoal-filtered cigarette smoke in the cytoplasm even after repeated exposures. Experiments are underway in our laboratory comparing the phagocytic ability of alveolar macrophages for bacteria in lung explant controls vs. cultures exposed to various doses of charcoal-filtered cigarette smoke.

The finding that the gas phase from charcoal-filtered cigarette smoke had a stimulating effect on DNA synthesis of alveolar macrophages but not epithelioid cells is also of special interest. It not only points again to the greater sensitivity of alveolar macrophages to factors from charcoal-filtered cigarette smoke, but also shows that particulate matter has a biological activity quite different from that of the gas phase. The two effects are indeed opposite and antagonistic ones,

that is, inhibition of cell metabolism by particulate matter, resulting in cell destruction; and stimulation of cell metabolism by the gas phase, resulting in increase of DNA synthesis.

The detection of these two different effects and their selective activity on alveolar macrophages was made possible by using cigarette smoke from which cytotoxic factors, such as acrolein, were eliminated by adsorption on activated charcoal (3,4,5). The presence of these cytotoxic factors masks other biological effects and their selective nature, in that unfiltered cigarette smoke results in a rapid generalized destruction of cultures, regardless of whether they contain mammalian or non-mammalian cells, such as yeast (3,4). It would thus appear that work on cultures with cigarette smoke from which these non-specific cytotoxic factors have been removed may be of help in attempts to detect and characterize different components in cigarette smoke which influence cell metabolism. This approach also seems fruitful in endeavours to elucidate the important and complex problems concerning the role and pathway by which cigarette smoke is implicated in malignant cell transformation.

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TUMOUR GROWTH IN RELATION TO THE IMMUNOLOGICAL ENVIRONMENT

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Malignant change in somatic cell populations may be a relatively common event (1) but the subsequent development of tumours may usually be prevented by the operation of immunological surveillance mechanisms (2,3). The immunological environment of the host may thus play an important role in the regulation of tumour growth and metastasis. Attempts to utilize immunotherapy to control the growth of tumours have, however, been unsuccessful (4,5), although recent attempts to stimulate host responses have achieved an encouraging degree of success (6,7). This has emphasized the urgent need for precise information about the immunological mechanisms and the cell populations which may influence tumour growth. We have been accumulating such information in a series of experiments in which factors affecting the growth of implants of a spontaneous murine mammary adenocarcinoma have been analysed (8,9).

THE GROWTH OF TUMOUR IMPLANTS IN SYNGENEIC RECIPIENTS

In CBA females or males aged between ten and sixteen weeks, the tumour grows well. The growth rate of the transplanted tumour is not affected by small variations in implant weight or by the time interval between implantation and harvesting of the tumour (10). The tumour generation can effect the growth rate of the tumour: earlier generations growing slower than later generations (9). The diameter of tumour implants of later generations (SMT 17 - 17th transplant generation) increases linearly with time and the tumour reaches sizeable proportions by the fourteenth day after transplantation (Fig. 1).

THE GROWTH OF TUMOUR IMPLANTS IN ALLOGENEIC RECIPIENTS

Untreated Recipients

Allografts of the CBA adenocarcinoma are briskly rejected in CS1 female recipients (Fig. 1). The tumour achieves only small proportions by about the eighth day after transplantation and then regresses. On histological examination six days after implantation, accumulations of cells around the periphery of the tumour are observed

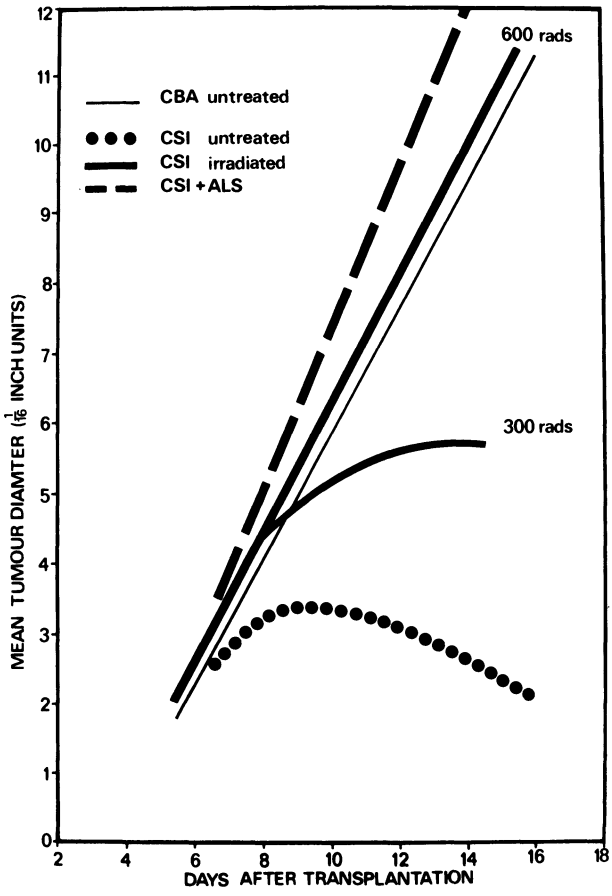

Figure 1. The Primary Response. The growth of implanted fragments of a spontaneous murine adenocarcinoma in syngeneic (CBA) recipients and in allogeneic (CS1) recipients. Allogeneic recipients included animals exposed to whole-body X-irradiation or treated with antilymphocyte serum.

although they do not appear to penetrate the tumour itself (10). CS1 mice that have been sensitized to a tumour allograft will reject a second implant of the tumour extremely briskly (Fig. 2).

X-irradiated Recipients

The immune response directed against allografts can be suppressed by exposing recipients to whole-body X-irradiation (11,12,13,14). For early transplant generations of the CBA adenocarcinoma, for

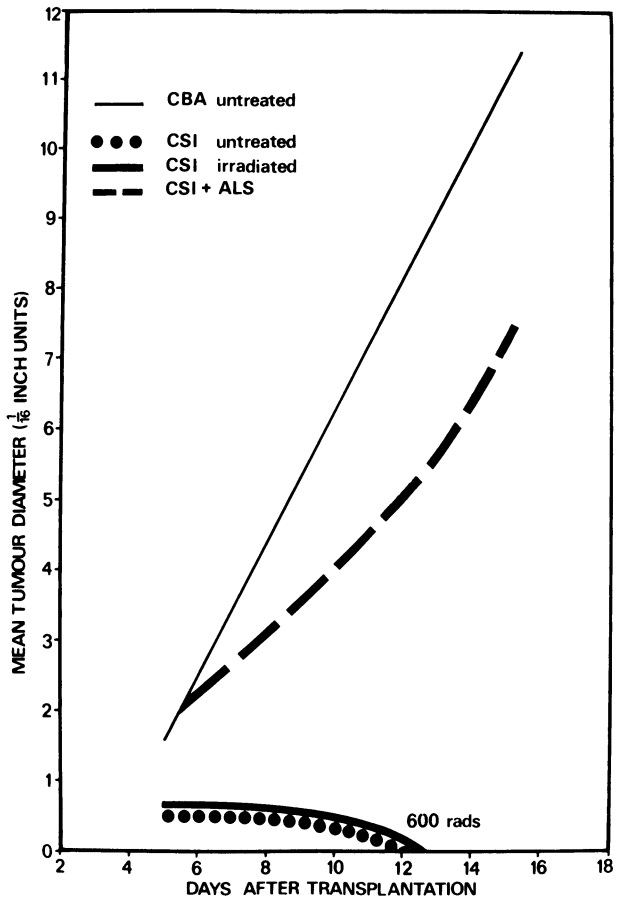

Figure 2. The Secondary Response. The growth of implanted fragments of a spontaneous murine adenocarcinoma in syngeneic (CBA) recipients and in allogeneic (CS1) recipients which had previously rejected an allograft of the tumour. Allogeneic recipients included animals exposed to whole-body X-irradiation or treated with anti-lymphocyte serum.

which the growth rate was slow, a marked dose dependency could be demonstrated between the diameter of the tumour on the twenty-first day after implantation and the dose of X-irradiation administered. This relationship is, however, obscured for later generations. The tumour grows well in allogeneic recipients exposed to 600 rads of whole-body X-irradiation. In recipients exposed to 300 rads, it grows more slowly (Fig. 1). In contrast, recipients that have previously rejected a tumour allograft still retain the capacity for allograft rejection even after exposure to 600 rads of whole-body X-irradiation (Fig. 2). This radioresistance of the secondary response persists for at least 7 months after primary sensitization (9).

Recipients Treated with Antilymphocyte Serum

Antilymphocyte serum (ALS) will suppress the primary response to skin (15,16) and to tumour allografts (17). Tumour allografts grow rapidly and reach sizeable proportions during the second week after transplantation (Fig. 1). In contrast to the dichotomy of susceptibility of the primary and secondary responses to X-irradiation. ALS suppresses both the primary and the secondary responses to tumour allografts (Fig. 2).

THE GROWTH OF TUMOUR IMPLANTS IN IRRADIATED ALLOGENEIC RECIPIENTS TREATED WITH HAEMATOPOIETIC CELL SUSPENSIONS

Lethally Irradiated Recipients

Following the intravenous injection of haematopoietic cell suspensions, cells of donor origin repopulate the haematopoietic and lymphoid tissues of lethally irradiated recipients (18). Barnes, Ford, Ilbery, Koller and Loutit (19) have compared the growth of a CBA lymphoma and a strong A sarcoma in irradiated recipients treated with syngeneic or allogeneic haematopoietic cell suspensions. They have described how while the CBA lymphoma killed all of the mice, the response to sarcoma A was partially recovered within six to seven weeks, but still not fully recovered after three or four months. Recovery occurred much faster if lymph-node cells were added to the bone-marrow inoculum (20). The response to tumour allografts in lethally irradiated recipients treated with spleen or foetal liver cells has been followed immediately after therapy by Feldman and Yaffe (21), who described progressive growth of the tumour in foetal liver chimeras while in spleen chimeras the results were variable.

a) Spleen. Recovery has been studied in lethally irradiated mice treated with spleen cells. The capacity for tumour allograft rejection in the irradiated mice recovers within one month in mice treated with 25 x 10^6 spleen cells, although allografts transplanted

immediately after cell therapy grow progressively. Spleen cells (26 x 10^6) from a donor that has previously rejected a tumour allograft can, however, transfer immunity to lethally irradiated recipients immediately. Lower doses of spleen cells (11 x 10^6) also inhibit the growth of the tumour (8).

b) Bone Marrow. Groups of CS1 mice were exposed to 820 rads of whole-body X-irradiation (300 KVp, 5 m.a., 5 mm. aluminium + 0.5 mm. copper filtration, dose rate 60 rads/min.) and injected intravenously with 5 x 10^6 CS1 bone-marrow cells. Allografts of the CBA tumour were implanted after 0, 1 or 3 months. The viability and growth properties of the different tumour generations used were

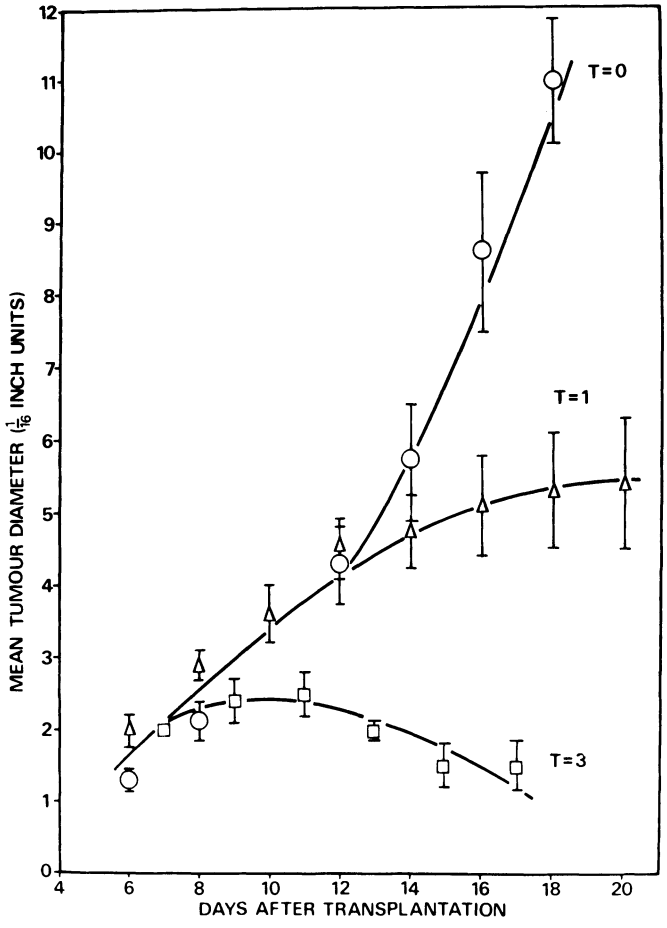

Figure 3. The growth of tumour allografts implanted at various time intervals after exposure of recipients to a lethal dose of whole-body X-irradiation (820 rads) and treated with 5 x 10^6 syngeneic bone-marrow cells (T=0, 1 or 3 months).

measured in CS1 mice corresponding in age with the experimental groups, which were exposed to 600 rads prior to implantation of allografts. The allografts grew well in the lethally irradiated recipients treated with bone-marrow when the tumour was implanted immediately after therapy (T=0). Recovery ensued, however, and within one month (T=1) the capacity for allograft rejection was partially restored and within three months (T=3) was completely restored (Fig. 3). There were no differences in the growth rates of the tumours implanted into the three groups of irradiated controls.

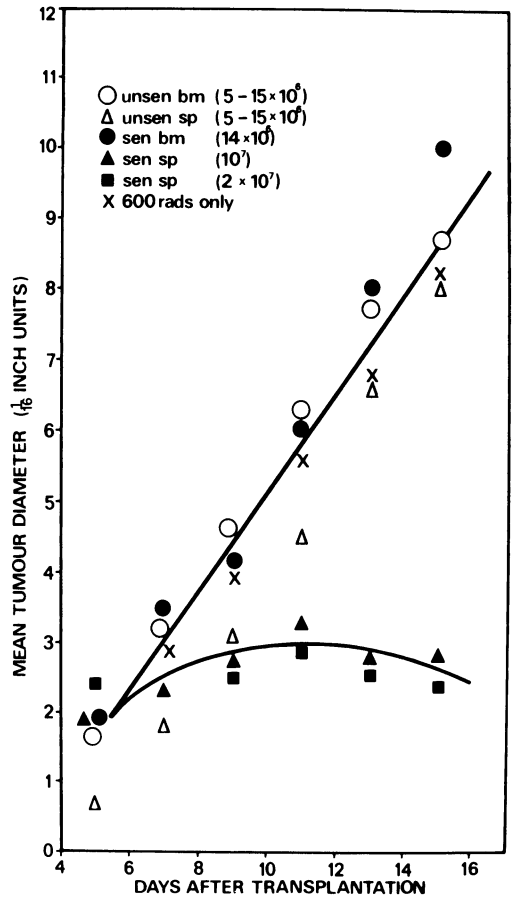

Figure 4. The growth of tumour allografts implanted after the exposure of recipients to a sub-lethal dose of whole-body X-irradiation (600 rads) followed by treatment with syngeneic bone-marrow or spleen cells from unsensitized donors or from donors which have previously rejected an allograft of the tumour (sensitized).

Sub-lethally Irradiated Recipients

Reduction of the period of progressive growth and of the time required for rejection of an A strain sarcoma has also been described following the administration of spleen and lymph node cells to sub-lethally irradiated C mice (22). Sensitized spleen and lymph node cells injected subcutaneously also influenced the growth and subsequently the rejection of a BP8 homograft in irradiated recipients treated with bone-marrow cells.

a) Spleen. Groups of CS1 mice were exposed to 600 rads of whole-body X-irradiation. One group was injected intravenously with 10^7 spleen cells from unsensitized donors, two other groups were injected intravenously with 10^7 or 2×10^7 spleen cells from sensitized donors and a further group was left untreated. Tumour allografts were implanted immediately after therapy. There was no difference in the growth rates of tumours in the irradiated animals which received no cell therapy and in those treated with spleen cells from unsensitized donors ($P=0.8$, day 15). The tumour allografts were all rejected, however, in the recipients which were treated with spleen cells from sensitized donors ($P<<0.001$, day 15 between unsensitized and sensitized spleen groups, Fig. 4).

b) Bone Marrow. Groups of CS1 mice were exposed to 600 rads of whole-body X-irradiation and injected intravenously with 10^7 bone-marrow cells from unsensitized donors or 14×10^6 bone-marrow cells from a donor that had previously rejected a tumour allograft. Tumour allografts were implanted immediately after cell therapy. The growth rates of the allografts in irradiated animals treated with bone-marrow cells from sensitized or unsensitized donors were not different from the growth rates of allografts in irradiated recipients which did not receive any cell therapy ($P=0.7$, day 15).

DISCUSSION

A model has been proposed to account for the establishment of immunity following the exposure of an organism to an antigen (23,24). Two cell compartments are postulated; one occupied by immunologically uncommitted progenitor cells (PC_1) and the other by cells derived from the first compartment following antigenic stimulation (PC_2). This model has been used successfully in the study of the humoral response (25,26) but its possible relevance to considerations of the relationship between a tumour and its immunological environment has not been fully explored (8,9). In the present study an attempt has been made to interpret impairment of the responses to tumour allografts by immunosuppressive agents in terms of a similar model.

As might be expected, tumour allografts are briskly rejected in

untreated mice. Transplantation of a second tumour is then followed by very brisk rejection due presumably to the establishment of a committed cell population (PC_2), which is suggested by the specificity of immunity to tumour cells (14,27). Enlargement of the progenitor cell population (PC_1) may also occur. Both irradiation (1,9,28) and ALS (9,29) can suppress the primary response by rendering impossible the necessary amplification or differentiation of the cells in the progenitor compartment. In contrast to the primary response, the secondary response is sensitive to ALS but not to radiation. The differences in the sensitivity of the secondary response to impairment by X-irradiation and by ALS therapy could of course result either from quantitative or from qualitative changes in the cells responsible for tumour rejection. Thus an enlarged population might be depleted below the minimum number of cells required to effect tumour rejection within two weeks following ALS therapy but not after doses of irradiation which can be employed in practice. Alternatively the cells of the committed pool (PC_2) established following rejection of a tumour allograft may be relatively more sensitive to ALS than to irradiation when compared with the cells from which they are derived (PC_1). Work on the humoral response suggests that the radiosensitivities of the cells involved in the primary and secondary responses are similar however, even though a new population of committed cells is established during immunization (30,31,32). In view of the association of immunosuppressive therapy with an increased tumour incidence (33,34,35,36) and the inadvertent transfer of malignant cells from a cadaver in a kidney graft (37) the present considerations should be borne in mind in the clinical utilization of ALS.

The failure of lethally or sub-lethally irradiated mice treated with spleen or bone marrow cells to effect tumour allograft rejection immediately after therapy may be attributed to the fact that the incompletely restored progenitor cell pool cannot undergo amplification and differentiation sufficiently rapidly to effect tumour rejection. Recovery ensues, however, and is complete within three months. The recovery of immune responses to skin allografts in bone marrow and spleen chimeras is complete within 28 days (38,39,40) and the humoral response in bone marrow chimeras is restored within two months (41). Although recovery of the capacity to reject tumour allografts occurs faster in the mice treated with spleen cells, it is difficult to compare the recovery patterns for the two donor tissues as the most meaningful criterion for comparison is difficult to define. A given number of bone marrow cells contains about seven times as many colony forming units (CFU) as the same number of spleen cells (10,42), so that animals treated with 5×10^6 bone marrow cells receive more CFU than animals treated with 25×10^6 spleen cells. As recovery occurs more rapidly in the latter group, it appears that the CFU content of a cell population is not the prime factor in determining its ability to restore the response to tumour allografts.

The ability of spleen cells from sensitized donors to effect rapid restoration of the capacity of an irradiated host to reject an allogeneic tumour, suggests that during the course of the tumour rejection in the donor, significant numbers of sensitized cells are produced in the spleen and are immediately effective following transfer or their precursors are increased in number so that the degree of amplification required can be readily achieved (8). Parallel changes in antibody forming potential have been reported by Nettesheim, Makinodan and Williams (25), who compared the ability of sensitized and unsensitized spleen cells to produce haemagglutinin in response to rat red blood cells following transfer to irradiated mice. Similar changes do not, however, occur in the bone marrow of mice that have rejected a tumour allograft. Observations on the recovery of immune responses in thymectomized recipients have previously indicated that the bone marrow contains few immunologically competent cells (43). In contrast to the inability of bone marrow cells to transfer the capacity to effect tumour rejection, bone marrow cells can transfer tolerance to irradiated recipients (44).

SUMMARY

Implants of a spontaneous murine adenocarcinoma, which grow rapidly in syngeneic recipients, are rejected briskly in allogeneic recipients. Following the administration of whole-body X-irradiation or of antilymphocyte serum to allogeneic recipients, this tumour grows as well as it does in syngeneic recipients. Animals which have already rejected a tumour are, however, able to reject a second implant despite irradiation but their ability to reject a tumour allograft is still abolished by treatment with antilymphocyte serum.

Following the intravenous injection of bone-marrow or spleen cell suspensions irradiated recipients gradually recover their capacity to reject tumour allografts. Animals treated with spleen cells from sensitized donors, which have previously rejected allografts of the tumour, are able to reject an implant immediately after being treated. Bone marrow cells from sensitized donors are in contrast no more effective than bone-marrow cells from unsensitized donors.

These observations can be usefully considered in relation to the model (23,24) which has been proposed to account for the development of immunity following the exposure of an organism to an antigen.

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MACROPHAGE RECOGNITION MECHANISMS: CLINICAL AND EXPERIMENTAL CONSIDERATIONS*

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Phagocytosis is an active process dependent upon both cellular activity (1-4) and humoral recognition factors (HRF) (5-10). The latter are essential for the expression of the discriminative actions of macrophages. Alterations in either the cellular or humoral components of the RES manifest themselves in altered intravascular clearance of colloidal materials (7,8,11,12). In addition to altered intravascular clearance rates, depression of reticuloendothelial (RE) function, by either alterations of cellular activity or depletion of HRF, is also associated with a decreased production of humoral antibodies (13,14) and cellular transplant reactions (15). Conversely, RES stimulation is associated with increased humoral (16) and cellular immunity (15). It is clear from these and other studies that the RES constitutes the afferent limb of the immune response.

Previous studies (17-20) have indicated that neoplasia is associated with a significant decrease in immunological competency. Grace and Kondo (17) have reported prolonged skin graft survival during the neoplastic disease state in man, while others (18,19) have demonstrated a defect in delayed hypersensitivity reactions. Since such cell-mediated immunological phenomena can be associated with altered RES phagocytic function (15,20,21), it would be reasonable to expect that either cellular or humoral activity of the RES is altered during neoplasia.

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Several investigators (22,23) have reported that overt RES activity is depressed during the neoplastic state as measured by intravascular clearance rates. In contrast, Salky et al. (24) reported a hyperphagocytic state during neoplasia. The test particle utilized in the studies of Salky et al. (24) was subsequently shown not to require prior reaction with HRF for its phagocytosis (25). The HRF requirements of the colloidal particles utilized by those investigators (22,23) reporting decreased RES function during neoplasia are not known.

Bennett et al. (26) reported that tumor cells must be exposed to serum before the macrophage could ingest them. It is, therefore, possible that a loss of HRF function during neoplasia would result in the inability of the host to recognize the altered-self or antigenic qualities of tumor cells. Neoplasia would, therefore, represent a macrophage HRF dysfunction disorder.

In view of our recently developed techniques to define cellular and humoral aspects of phagocytosis (6-9,14-16,24,25, 27), studies were undertaken to evaluate the status of HRF in patients with neoplasia. In view of the observation of profound depletion of HRF activity in human neoplastic conditions, studies were undertaken to evaluate HRF alterations in an animal leukemia model.

METHODS

Blood was obtained via venipuncture from healthy control subjects, carcinoma patients, and patients with non-cancerous disorders.

Serum HRF activity was determined by the method developed by Di Luzio and colleagues (7,25,27). Briefly, rat liver slices (approximately 250 mg) were prepared with a Stadie-Riggs tissue slicer and placed in Erlenmeyer flasks which contained either 3 ml of Krebs-Ringer phosphate buffer, serum, or dilutions of serum in Krebs-Ringer phosphate buffer. Gelatinized RE test lipid emulsion labeled with I^{131} triolein (28) was added to the flasks to a final concentration of 2 mg of emulsion per flask. Finally, 100 U.S.P. units of heparin, which has been shown to be essential for Kupffer cell phagocytosis with either rat or human serum (25,27-29), were added in the amount of 100 units to each flask. The flasks were gassed with 95% O_2 and 5% CO_2 and incubated for 30 min. at 37°C in a Dubnoff metabolic shaker. The amount of I^{131}-labeled lipid emulsion phagocytized by the liver slices was determined with a Nuclear Chicago Auto-Gamma Scintillation Counter following two saline washes. Results are expressed as the per cent of the injected dose of lipid emulsion phagocytized per 100 mg of liver tissue (%ID/100 mg).

All human serum samples were assessed in duplicate for HRF activity at varying serum concentrations. At the time of HRF assay no knowledge of the clinical state of the subjects was made available to the individuals conducting the assay.

Peritoneal macrophages were obtained by introducing into the peritoneal cavity of rats 20 ml of a 12% sodium caseinate solution. Four days later the peritoneal cavity was washed with isotonic saline and the cells isolated by centrifugation. The peritoneal cells were washed three times with Krebs-Ringer phosphate buffer and adjusted to a concentration of 10 x 10^6 cells per ml. To 1.0 ml of cells was added 100 U.S.P. units of heparin, 1.0 ml of either control human serum or serum derived from patients with carcinoma and 2 mg of I^{131}-triolein labeled gelatinized RE test lipid emulsion. The cell suspension was then incubated, with constant agitation, at 37° C for 30 min. Following incubation, the cells were washed 3 times with physiological saline and cell radioactivity determined. All incubations were conducted in quadruplicate, means values determined and data expressed as the per cent of control.

Similar studies were undertaken in which human pulmonary macrophages were obtained by saline lavage of a lung obtained at autopsy. The subject was a 10 year male who died of accidental injuries. Cell viability of the human alveolar macrophages, which approximated 95%, was ascertained by trypan blue exclusion. Oxygen uptake, determined by polarographic techniques, further denoted the physiologic state of the isolated macrophages.

Long-Evans rats were employed as host animals for chloroleukemia tumors.* Tumors were maintained by the subcutaneous injection of 10^6 tumor cells into 50 g rats. The induction of leukemia in experimental animals was accomplished by the intravenous injection of 10^6 tumor cells into rats weighing approximately 250 g. In the studies which correlated peripheral leucocyte levels with humoral recognition factor activity, control animals received 10^6 normal white blood cells, obtained from the buffy coat of normal Long-Evans rats. Humoral recognition factor activity was evaluated in control and leukemic cell transplanted rats at frequent intervals as were blood leucocyte levels.

*Shay Chloroleukemic tumor bearing Long-Evans rats were initially provided by Dr. Eugene Handler of Hunter College.

RESULTS

Normal control human serum promoted the *in vitro* phagocytosis of gelatinized RE test lipid emulsion by rat liver slices to a comparable degree as rat serum (Table 1). In the absence of serum, the liver slices removed only 0.6% of the added lipid emulsion. The addition of serum to the media induced the phagocytic event in proportion of the amount of serum added; thus, a two-fold increase in phagocytosis occurred when the serum concentrations were doubled from 16.7% to 33.3%. In the presence of 100% normal control human serum, approximately 30 times as much lipid emulsion was removed by the Kupffer cells of the liver slices as was phagocytized by liver slices incubated in buffer alone. In all studies, normal rat serum or normal human serum was employed as a control in the evaluation of serum recognition activity. The rat serum control was utilized to assure that any phagocytic alterations noted with human serum was a function of the serum and not due to cellular activity of the liver slice which was employed.

Patients with non-metastatic carcinoma were initially used as serum donors for HRF assay (Table 2). None of the patients had as yet received any therapy for cancer.

Serum derived from patients with non-metastatic carcinoma manifested significant depression of HRF activity when employed as the incubation media for rat liver slices (Table 2). In a media of 100% serum from carcinoma patients, rat liver slices phagocytized

Table 1: Comparative humoral recognition factor activity of serum obtained from normal rats and normal human control subjects as determined by the degree of phagocytosis of gelatinized RE test lipid emulsion by rat liver slices.[a]

Incubation Medium	SERUM CONCENTRATION				
	0.0	16.7	33.3	66.7	100.0
	Phagocytic Uptake, %ID/100 mg				
Human Serum	0.6 ±0.06	4.5 ±0.8	9.9 ±1.2	16.3 ±1.8	19.6 ±1.4
Rat Serum	0.6 ±0.04	3.5 ±0.3	7.5 ±1.2	15.5 ±1.6	17.2 ±1.4

[a]Values, expressed as mean ± standard error, are derived from 32 animals. The normal human group consisted of six male and two female subjects. Ages ranged from 24 to 75 years.

Table 2: Humoral recognition factor activity of serum obtained from patients with untreated non-metastatic or metastatic carcinoma as compared to patients which received treatment or those with other disease states.

Group	SERUM CONCENTRATION 0.0	16.7	33.3	66.7	100.0
	Phagocytic Uptake, %ID/100 mg				
Normal Control	0.6 ±0.06	4.5 ±0.8	9.9 ±0.2	16.3 ±1.8	19.6 ±1.4
Carcinoma patients[a]	0.7 ±0.01	2.3 ±0.4	2.8 ±0.8	2.6 ±0.6	3.1 ±0.8
Metastatic Carcinoma patients[b]	0.6 ±0.01	1.8 ±0.4	2.1 ±0.5	1.3 ±0.3	1.0 ±2.0
Carcinoma patients in therapy[c]	0.7 ±0.01	2.7 ±0.4	4.2 ±0.4	5.8 ±0.6	8.7 ±0.8
Non-neoplastic disease group	0.6 ±0.06	4.2 ±0.8	9.9 ±1.6	11.8 ±1.5	13.5 ±0.6

[a]Carcinoma patients were four males and four females, ranging in age from 40 to 75 years. The site of lesion was liver (2), lung (4), and colon (2).

[b]Metastatic group consisted of five females and four male subjects ranging in age from 54 to 78 years. Liver and intra-abdominal involvement generally characterized this population.

[c]Group, consisting of five subjects, was assayed 1 to 9 days following treatment which consisted of cobalt irradiation and/or surgical removal of the tumor.

[d]Non-cancerous group consisted of 9 males, ages ranging from 42 to 61 years. Clinical disorders include such diverse entities as diabetes, hypertension, gastric and duodenal ulcers, pneumonia, emphysema and hepatitis.

approximately 3.0% of the gelatinized lipid emulsion, representing a mean 84% reduction in the ability of serum to enhance phagocytosis. Studies with serum from 9 patients with metastatic carcinoma revealed essentially the same findings (Table 2). Serum obtained from metastatic carcinoma patients possessed HRF activity equivalent to 5% of normal human serum.

Since the 17 patients with various carcinomas described above manifested a highly significant loss in serum recognition mechanisms prior to any cancer therapy, the effects of cancer therapy on the activity of HRF was evaluated. Five patients were used as serum donors (Table 2) and the ability of their serum to enhance phagocytosis was determined.

All of the 5 patients who had received therapy manifested a rather prompt elevation in HRF activity (Table 2). The average phagocytic uptake of lipid emulsion that was supported by the treated patients was 280% higher than that supported by serum from donors with untreated, non-metastatic carcinoma and 870% higher than serum from donors with untreated, metastatic carcinomas.

Since it is conceivable that disease states in general could produce a decrease in HRF activity and, therefore, the results reported here would not be significant to the neoplastic process, per se, a group of 10 patients with various disease states was employed for assessment of HRF activity (Table 2). There was no significant difference between the activity of the serum derived from the non-cancerous control subjects and the normal control subjects except for a slight reduction observed when a 100% serum was employed.

In vitro phagocytosis of gelatinized RE test lipid emulsion by isolated peritoneal macrophages was also depressed when serum derived from cancer patients was employed as the incubation media (Table 3) denoting the reduction in HRF activity was not a unique feature of the liver slice system. While normal human and rat serum each promoted about a 10-fold increase in phagocytosis as compared to the phagocytosis in buffer media, serum derived from patients with carcinoma had minimal phagocytic promoting ability. Indeed, the 85% reduction in its ability to promote phagocytosis rendered the plasma of patients with carcinoma almost equivalent to the buffer medium.

When HRF activity was evaluated using human alveolar macrophages and the gelatinized RE test lipid emulsion, the uptake of the gelatinized lipid emulsion by human alveolar macrophages was enhanced approximately 4-fold when plasma was present (Table 4). Human plasma was again as effective as rat plasma in supporting the uptake of the particle. In marked contrast, plasma obtained

Table 3: Inability of serum derived from patients with carcinoma to promote the phagocytosis of gelatinized RE test lipid emulsion by isolated rat peritoneal macrophage.

Medium	No.	Phagocytic Uptake (%ID/100 mg)
Buffer	18	2.3 ± 1.9
Normal rat serum	15	24.4 ± 1.1
Normal human serum	8	28.9 ± 3.5
Human serum Carcinoma patients*	5	4.4 ± 1.5

Values are expressed as means ± standard error.

*The carcinomatous subjects were three males and two females, ages ranging from 41 to 58. The area of involvement was breast (1), stomach (2), rectum (1) and colon (1).

Table 4: Assay of humoral recognition factor activity by employment of human alveolar macrophages

Incubation Media	Phagocytic Uptake[a]
Buffer	12.7
Rat plasma	46.3
Normal human plasma	45.4
Plasma, Breast carcinoma patient	14.3

[a]All values are the mean of 4 determinations with values expressed as per cent of the gelatinized RE test lipid emulsion phagocytized per 4 x 10^6 cells.

from a patient with carcinoma, did not materially support the phagocytosis of the gelatinized RE test lipid emulsion by human macrophages.

It is possible that a circulating plasma inhibitor of macrophage function, or recognition factor-particle interaction rather than a decrease in HRF activity could be the mechanism of inability of serum from cancer patients to support phagocytosis. To evaluate this possibility, liver slices were incubated in a mixture of 50% normal human serum and 50% serum from cancer patients to determine if the latter serum would impair the normal phagocytic-promoting activity of human serum. Gelatinized RE test lipid emulsion was employed as the particle (Fig. 1). The addition of serum derived from cancer patients to the serum of normal individuals did not

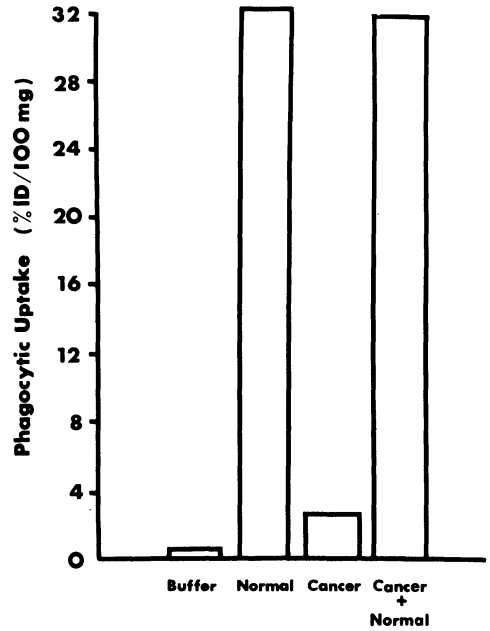

Fig. 1 Lack of a macrophage phagocytic inhibition factor in serum derived from patients with carcinoma. Liver slices were incubated with gelatinized RE test lipid emulsion in either buffer, 50% normal human serum in buffer, 50% cancer patient serum in buffer, or 50% normal human serum in cancer patient serum and 100 U.S.P. units of heparin. Each value represents the mean of 8 determinations.

depress the uptake of the lipid emulsion. In contrast to the 32% uptake manifested by liver slices incubated in normal serum, or the 31% uptake in the presence of normal serum plus serum derived from cancer patients, liver slices incubated in cancer serum alone never removed greater than 3% of the added lipid emulsion from the media.

In an effort to develop an experimental model to the clinical counterpart, rats were transplanted with either normal leucocytes or neoplastic leucocytes. In rats which received intravenous injections of normal white cells, no significant alteration was observed in either plasma humoral factor activity or in blood leucocyte levels (Fig. 2). The stability of plasma humoral recognition factor activity with time is clearly manifested in the control group.

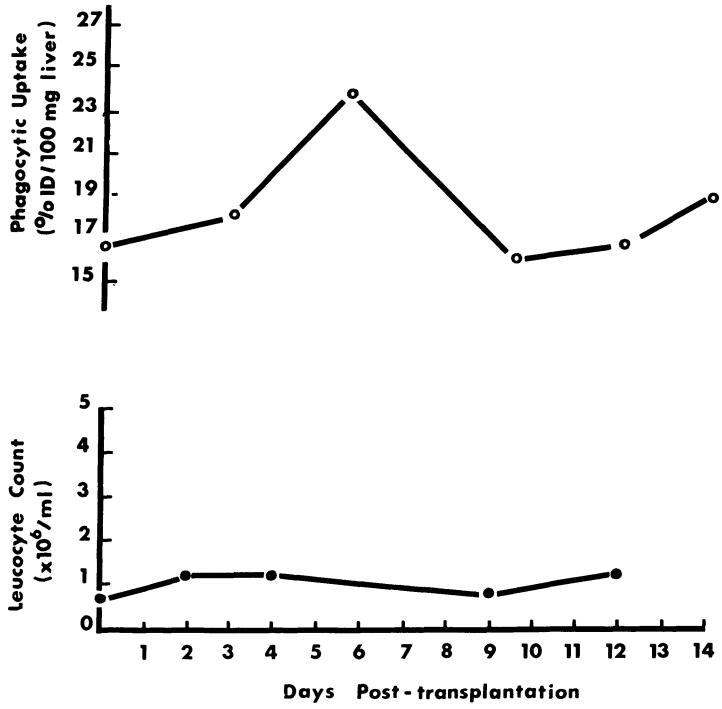

Fig. 2 Humoral recognition factor activity and white blood cell counts of rats determined at various intervals following the transplantation of 10^6 normal white blood cells obtained from isologous rats. Each point represents the mean of 22 determinations.

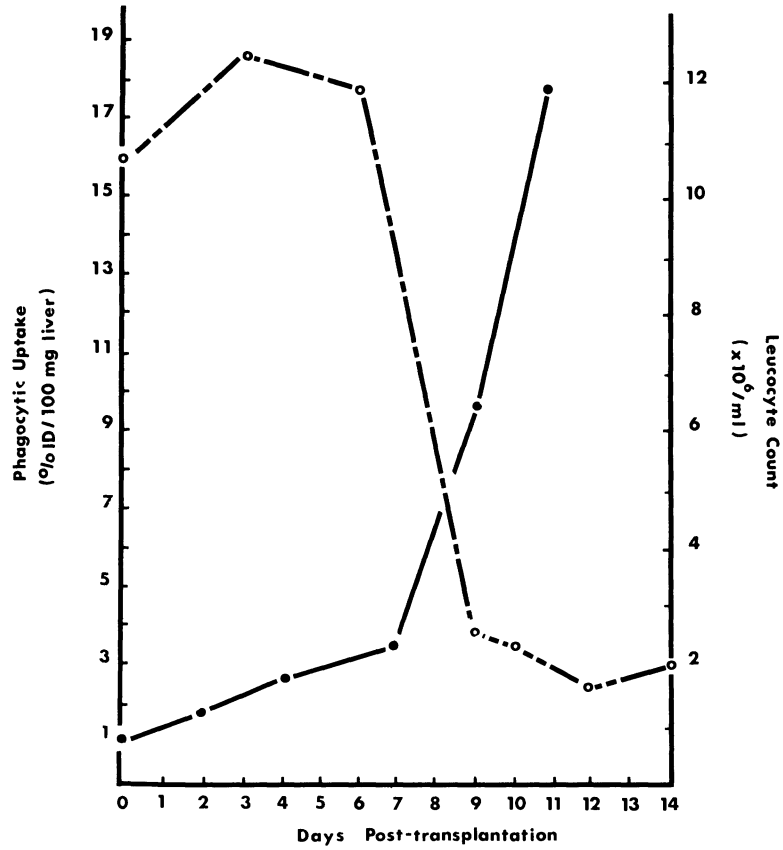

Fig. 3 Relationship between leukemia (-0-) and humoral recognition factor activity (--0--). Each point represents the mean of 10 determinations and intervals are days post transplantation of 10^6 leukemic cells.

In marked contrast, in rats which developed acute myelogenous leukemia (Fig. 3), plasma recognition factor activity was depressed approximately 80% at the ninth day following transplantation. The depression in HRF activity appeared to occur prior to the rapid phase of tumor growth are reflected by peripheral leucocyte levels, suggesting a possible cause and effect mechanism.

DISCUSSION

Human serum was as capable as rat serum in serving as a source of HRF in the rat liver slice system. The inability of the Kupffer cells of the liver slice to phagocytize the gelatinized RE test lipid emulsion particles in the absence of

serum has been shown to be related to a lack of humoral "non-self" recognition mechanisms (7,8,28). The dependency of nearly all fixed phagocytic cells upon serum factors has been previously documented (5-10,25-29). Indeed, recent reports have demonstrated a clinical syndrome in newborns which results in increased susceptibility to infection and is associated with specific depletion of opsonic activity (30,31). Thus, both clinical and experimental evidence exist for the dependency of the RES upon HRF activity in host-defense physiology.

Since the injection of large amounts of foreign colloidal material into an experimental animal effectively depletes the host of HRF activity (7,8) and results in a condition of reticuloendothelial depression, one would expect an impairment of phagocytic activity in clinical situations during which the host is exposed to a large population of foreign cells, such as bacterial, and perhaps tumor cells which possess tumor specific antigens. Indeed, Hektoen (32) demonstrated that the opsonic mechanisms of serum are depleted during bacteremia and unpublished observations from our laboratory have confirmed this early finding of Hektoen in that patients with fulminating tuburcular lesions were shown to lack HRF activity similar to the conditions described during neoplasia.

Evidence has accumulated which demonstrates that the surface configuration of many cancerous cells are altered in such a manner as to render the tumor cell "non-self" (17,33-35). Thus, cancerous cells might be expected to be removed from the circulation by the process of phagocytosis following reaction with HRF. Tumor growth and development may well be indicative of a failure in either the phagocytic ability of the host macrophages or the loss of HRF mechanisms resulting in loss of functional immunosurveilance. Based upon the present experimental and clinical observation, it is apparent that neoplasia is associated with the effective establishment of HRF-induced impairment.

One alternative to the concept that HRF depletion occurs during neoplasia would be that a substance is secreted in response to, or by the tumor cells which act as an inhibitor to either macrophage activity or to HRF. If a cellular inhibition factor were the explanation for the inability of serum from neoplastic patients to support liver slice phagocytosis, then it would be expected that intravascular clearance of colloids, which do not require HRF, would be impaired. While several investigators have reported that cellular inhibition occurs (36,37), the observations of Salky *et al.* (24) and Sheagren *et al.* (39) which demonstrated enhanced intravascular clearance of colloids that do not require the presence of HRF (28), would not support the concept that neoplasia is associated with the production of a macrophage

inhibition factor. In addition, the observation that serum derived from cancer patients, when mixed with normal human serum, did not inhibit the usual enhancement of phagocytosis observed with the normal human serum is contrary to the concept of the presence of an inhibitor.

The present studies have also demonstrated that the HRF activity from serum of neoplastic patients tends to increase following either radiation or surgical treatment. The ability of patients to recover HRF levels in as short a time as 24 hours following surgery is not too surprising. Rats which have been "blockaded" with gelatinized RE test lipid emulsion manifest total recovery of HRF activity within 4 hours post-blockade (7,8), indicating rapid synthetic mechanisms which are responsible for maintaining normal HRF levels in the plasma (7) and clearly denote the existence of a control mechanism. Our findings, therefore, suggest the possibility of a tumor factor which either impairs HRF synthesis or function or that HRF is depleted due to the presence of tumor cells. It is also possible that an aberrant HRF is formed which complexes with the tumor cell, but does not promote its phagocytosis.

Previous investigators have reported that the RES is depressed in neoplastic patients when either colloidal congo red (22) or aggregated albumin (23) were used as test particles for intravascular clearance. The studies reported here would seem to support these conclusions. However, Salky et al. (24), employing non-gelatinized RE test lipid emulsion as test colloid, found a hyperphagocytic RES during neoplasia, as did Sheagren et al. (38), when intravascular clearance rates were compared to normal subjects. Previous studies in experimental animals have indicated that during states of HRF depletion, the Kupffer cells of the liver demonstrate hyperactivity (7) and undergo hyperplasia (40). The latter is also a feature of neoplasia. It is possible that the hyperactivity reported by Salky et al. (24) and Sheagren et al. (38) can be attributed to a hyperactive cell population within the RES and an increase in the bulk of the RES cellular elements due to hyperplasia and, thus, bears no relationship to the HRF activity of serum. Hyperactivity and hyperplasia of the macrophage cell population are especially relevant to the studies of Salky et al. (24) and Sheagren (38) since these investigators employed particulate preparations which do not appear to require prior interaction with HRF for its phagocytosis (25). A similar situation can be shown for infectious patients where serum recognition factors are depleted during infection but cellular hyperactivity occurs (41).

A major question is whether or not tumor cells require HRF for their phagocytosis, i.e., are tumor cells foreign, or in

essence "altered self"? While this point is yet to be fully established, Bennett et al. (26) have demonstrated that certain tumor cells cannot be ingested by peritoneal macrophages in the absence of normal serum, thus indicating recognition factor requirement for the phagocytosis of a variety of tumor cell types. The ability of tumor cells to pass through the liver, or to reside within the liver, and escape phagocytosis and destruction by liver Kupffer cells (42) is highly indicative of a phagocytic defect in the host.

Further evidence for both the "non-self" or "altered-self" attributes of tumor cells, as well as the inability of patients with neoplasia to manifest normal rejection mechanisms was demonstrated by Southam et al. (43) and Levin et al. (44). Southam and colleagues (43) demonstrated rejection of cancerous tissue cell lines by normal individuals but a profound decrease in such rejection mechanisms in patients with neoplasia (43). Levin et al. (44) confirmed and extended the findings of Southam et al. (43) by demonstrating that in addition to normal individuals, patients with advanced, debilitating, non-neoplastic diseases also had the ability to reject tumor cells while cancer patients have a profound defect in their ability to reject foreign tissue transplants. Since Southam et al. (43) also demonstrated that patients with neoplasia have normal antibody-producing mechanisms, it appears that the loss of tissue-rejection mechanisms is a specific defect of normal cellular immunity. Such a loss of cellular immune phenomena might be attributed to the inability of the macrophage to recognize foreign materials, i.e., a loss of HRF activity rather than a cellular impairment.

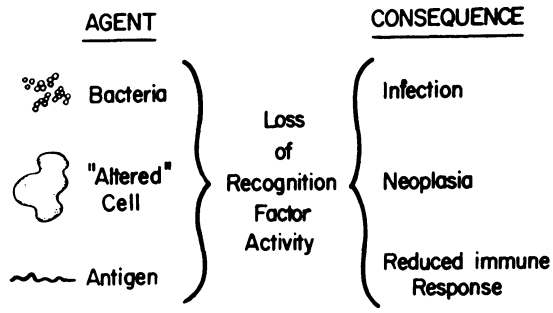

Fig. 4 Postulated importance of loss of recognition factor activity by which macrophages detect the presence of foreign particulates in their environment to the development of a disease state. This working hypothesis is presently under active investigation and evaluation.

It would appear that one major defect in patient with neoplastic disease is the loss of HRF activity. The relationship between HRF and neoplasia cannot be fully ascertained at this time, although experimental studies of acute myelogenous leukemia are suggestive of a close relationship. This relationship is also established by the recovery of HRF activity upon treatment. It is considered possible that the presence of foreign tumor cells results in a depletion of HRF and a subsequent establishment of an HRF mediated reticuloendothelial impairment (Fig. 4). Hence, immunocompetency and tumor rejection mechanisms which are seemingly depressed during neoplasia may well be the result of HRF depletion which would then be a primary event of the neoplastic process.

SUMMARY

The expression of the phagocytic event by macrophages requires that certain humoral recognition factors (HRF) be present in order for macrophages to discriminate between "self", "altered-self" and "non-self". Kupffer cells of rat liver slices, in an incubation medium consisting of rat serum and heparin, manifested a 17-fold enhancement in uptake of gelatinized RE test lipid emulsion/100 mg of tissue as compared to the degree of phagocytosis manifested in buffer medium. Normal human serum, in the presence of heparin, was as effective as rat serum in promoting phagocytosis by rat liver slices,indicating that human HRF can also enhance the phagocytic activity of rat macrophages. In contrast, liver slices incubated in serum derived from 17 patients with untreated carcinoma, both metastatic and non-metastatic, manifested a mean 90% reduction of particle uptake, denoting loss of HRF activity. Recovery of HRF activity was observed following either radiation or surgical therapy. Patients with other clinical disorders were found to have essentially normal HRF activity, hence, the loss of HRF activity during neoplasia appears to be a phenomena associated with neoplasia. Comparable results were obtained when rat peritoneal or human alveolar macrophages were employed. Experimental studies in rats employing Shay Chlorleukemia transplants demonstrated marked depletion of HRF activity in plasma which coincided with the onset of leukemia. These findings denote that neoplastic disease in man and in rats is associated with profound decreases in HRF activity. In view of the partial restoration of HRF activity with treatment and the relationship of HRF activity to the development of leukemia, it is suggested that humoral recognition factor activity may be an important element in neoplasia.

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VIRUS INDUCED MALIGNANT LYMPHOME IN MICE DEPENDENT ON A RES 'CONDITIONED' BY CHRONIC PARASITIC INFECTION (*P. BERGHEI*)

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Burkitt's lymphoma (BL) is known to be common in regions where holo-endemic or hyperendemic malarial conditions exist. It has been postulated (1) that on the base of intense and prolonged malarial infection the lymphoreticular system (LRS) may be prone to malignant change in the presence of, or induced by, some viruses. This hypothesis would be supported by the finding that all BL patients have high antibody titers to the Epstein-Barr virus (2), and that the EB virus could be demonstrated in tissue cultures set up from Burkitt lymphomas (3).

In previous papers (4, 5) we described the appearance of an aleukemic, malignant lymphoma resembling BL in Swiss mice. The lymphoma was observed much more frequently in mice immune to *Plasmodium berghei* than in controls of the same age. Although numerous virus particles were seen to be associated with this disease, hithertoo the oncogenic properties of these particles have not been demonstrated. Therefore, investigations were carried out to solve the problem whether the aleukemic lymphoma can be regarded as a prolonged preleukemic stage, or as an independent tumor developing on the basis of a LRS "conditioned" by chronic malarial infection.

MATERIALS AND METHODS

In a first series of experiments (I) the influence of slight, moderate and severe malarial infections on the frequency of spontaneously, developing, solid lymphomas was investigated (fig. 1). Swiss mice, aged 85 to 95 days, and *Plasmodium berghei*, strain 173 K, were used for these experiments. Group I A (250 mice) served as controls. A group of 250 mice (I B) was kept on a para-aminoben-

zoic acid free milk diet for 10 weeks. These mice were inoculated 6 times (once a week) with 10^4 parasites to induce a controlled, low parasitaemia. Mice of group I C (450 animals) were infected with 10^6 parasites and 7 days later treated with milk diet. In this group parasitaemia lasted about 6 weeks and the maximum parasitaemia was about 30 per cent of rbc infected. Mice of group I D (1,400 animals) were infected with 10^6 parasites and 14 days later treated with milk diet. In this group parasitaemia lasted about 6 weeks and reached a maximum of 60 per cent. Surviving mice of group I D were challenged 3,5 and 7 months after the first inoculation.

Seven months after primary inoculation, i.e. at an age of 10 months, the surviving mice of each group were divided into two subgroups. Each subgroup a) consisted of 100 animals which were used for registration of spontaneous mortality. Mice of subgroup b), i.e. 130 animals of I A-b and I B-b, 210 animals of I C-b and 330 mice of I D-b, were used for histopathological investigations. Every second month, starting at an age of 10 months, 10 mice of each subgroup b) were selected at random, sacrificed and the thymuses, spleens, lymph nodes, and liver examined by gross appearance and by means of light microscopy and electronmicroscopy. Preparations of the groups I A-b and I D-b were routinely investigated for the presence of virus particles.

In a second series of experiments (II) the transplantability of aleukemic lymphomas was studied.
About 1,000 Swiss mice aged 53-58 days, were infected with *P. berghei*, strain 173 K, and kept on standard diet. Fourteen days after infection, when the mice had developed a mean parasitaemia of about 50%, the standard diet was replaced by a diet free of paraaminobenzoic acid (PABA). In surviving animals parasitaemia decreased about 10 days later, and 32 days after treatment with PABA free diet the mice were found to be free of parasites. Two months after inoculation with malarial parasites, i.e. at an age of 4 months, these mice were divided into 2 groups. One group (II A) of 150 animals served as controls. The other group (II B, 156 mice) was treated with 0,1 ml of a cell free extract prepared from lymphoma tissue. The cell free preparation of lymphoma tissue was also administered to 156 normal Swiss mice, aged about 4 months which had not endured malarial infection (group II C). One group of untreated Swiss mice served as controls (II D).

For the preparation of cell free tumor extracts, a modified polyribosome isolation procedure was applied. Pooled lymphoma tissue from the spleen, liver and thymus of 13 lymphoma bearing animals was dissected and homogenized with a Teflon-glass homogenizer in 9 parts isotonic Difco M 199/sucrose solution to 1 part tumor tissue. Subsequently, the homogenate was centrifuged at 4°C at 1,500 *g*. for 10 min. to remove nuclei and structured intercellular material. The supernatant was centrifuged again at 4°C at 10.000 *g*. to remove mitochondria and membrane complexes. The submitochondrial

fraction was used for administration to groups II B and II C.

Three months after the inoculation of the cell free tumor preparation 36 mice of group II B and II C, and 40 mice of the control groups II A and II D were selected at random, sacrificed, and the thymuses, spleens, and livers examined by gross appearance and by means of light microscopy and electron microscopy. At this time the mice were aged 7 months. Five months later 80 mice of group II A and II D, as well as surviving mice of group II B (62) and II C (73) were sacrificed and examined.

RESULTS

Results of experiments I are shown in fig. 1. A striking increase in the number of lymphoma carrying animals could be observed from group I A to I D indicating a relationship between the severity of endured primary malarial infection and lymphoma growth. The overall number of lymphoma bearing animals was 6.25% in group I A (controls), 10.0% in group I B (controlled low parasitaemia), 17.5% in group I C (moderate malarial infection), and 30.0% in group I D (severe malarial infection). Furthermore, aleukemic solid lymphomas were seen developing earlier in group I C and particularly in group I D than in groups I A and I B. Whereas there was no significant difference in the frequency of common murine leukemias in the groups I A to I D (11.25%, 12.5%, 11.5%, 10.0%), the number of mice carrying epithelial malignomas and sarcomas decreased from group I A to I D (11.25%, 7.5%, 6.25% and 5.0%).

Mice which had suffered from severe malarial infections (I D) showed a much higher spontaneous mortality, i.e. 68%, compared with the controls (23%). Virus particles were found particularly during the early stages of lymphoma growth, but were generally more frequently observed in group I D than in the control group I A. The predominant virus particles were of the A_1 and C type; they were either extracellular or located in enlarged intracellular vacuoles. In a few instances A_2 particles seemed to be located intracellularly. Only a few virus particles were seen budding off from immature and undifferentiated cells of the lymphoreticulocytic series. The majority of virus particles were found to be attached to, or budding off from cells of the differentiated reticular type. These reticular cells showed intense vacuolization and a disintegration of the cytoplasmic surface in most instances.

Results of experiments II. Three months after the inoculation of a cell free tumor extract 13.9% of the sacrificed mice of group II B and 2.8% of group II C showed aleukemic lymphomas. No lymphoma carrying animals were found in groups II A and II D. Eight months after the inoculation of a cell free tumor extract into mice which had endured malarial infection (group II A) 59,7% showed aleukemic lymphomas. In group II C (inoculation of the tumor extract into normal mice) 32.8% were found to be diseased. Spontaneously

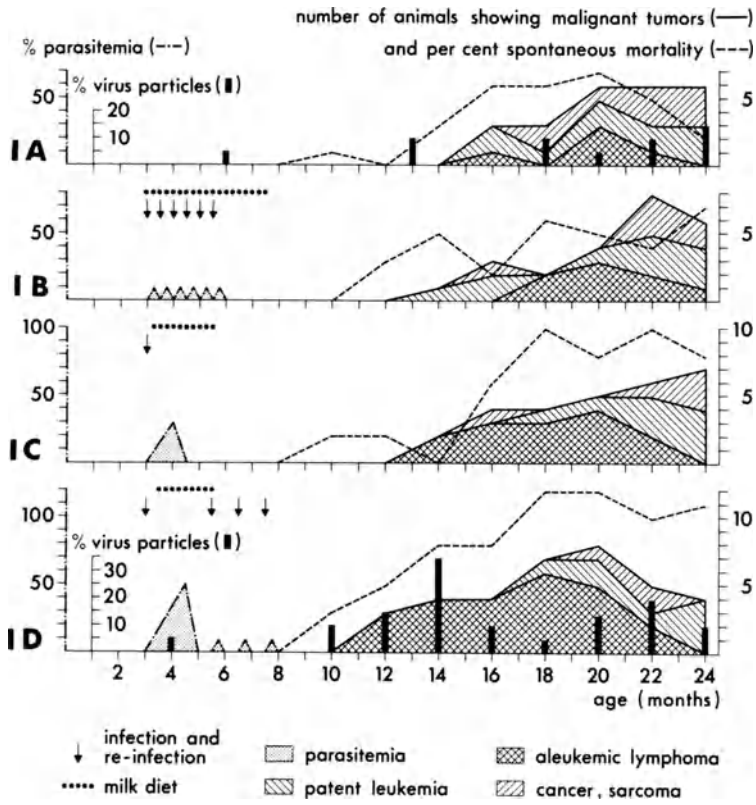

Fig. 1. Frequency of spontaneously developing aleukemic lymphomas, common murine leukemias, and other malignant tumors in control animals (I A) in mice after controlled low parasitaemia (I B) after moderate (I C) and severe malarial infection (I D).

developing aleukemic lymphomas were observed only in group II A, which had suffered from severe *P. berghei* infection; ten per cent of these mice carried lymphomas.

It should be noticed that in group II B 16.1% presented the picture of typical erythroid and myeloic leukemias, while the incidence of these leukemias in group C was 24.7%.

Conspicuous differences were observed in organ distribution of transplanted lymphomas in groups II B and II C. Whereas tumors transplanted to normal mice (group II C) involved the thymus in every case (100%), and in 29% the spleen, but in no instance the liver, lymphomas developed following inoculation of cell free tumor extracts in the of 33% of mice which had endured severe malaria infection. In this group(II B)the liver was found to be involved in 51.3%, of the affected mice, whereas the incidence in the thymus was only 86.2%.

Light microscopically, the transplanted tumors showed the same appearance that described in a previous communication (4). The predominant cell type was an immature, small, medium sized, or large "round cell" which exhibited under the electron microscope numerous regularly distributed and rosette-like arrays of free polysomes. Only a few cells showed a rough endoplasmatic reticulum. Histiocyte-like cells, as well as mon- and polynucleated giant cells were regularly distributed, causing the familiar "starry sky" appearance in many instances. In the thymus the tumor was infiltrated by eosinophillic erythrocytes.

As pointed out in a previous paper (5), in the thymus of mice which had suffered from malarial infection lymph follicles with germinal centers could be observed. These germinal centers appeared to be the foci of lymphoma growth as judged from their rapid enlargement. The same dynamic changes were observed in the spleen. In some cases the initial lymphoma growth was associated with an increase in number of mature plasma cells, in the spleen as well as in the lymph nodes. In the liver the aleukemic lymphoma developed only in the periportal areas.

Numerous virus particles were seen in association with tumor growth, mostly C-particles, and in some cases A_1 particles as well as A_2 particles were found. Surprisingly, most particles were found to be attached to, or budding off from cells of the reticular type. In the thymus virus particles were almost exclusively seen budding off from epitheloid cells.

DISCUSSION

Two theories have been considered concerning the pathogenesis of Burkitt's lymphoma: 1) A primary oncogenic virus transmitted by a vector; 2) A primary nonmalignant virus (e.g. EB virus), capable of transforming lymphoreticular cells previously "conditioned" by chronic parasitic infection. Results of our experiments support the second theory. Swiss mice do not only develop solid lymphomas after severe infection with *P. berghei* to a much higher percentage than controls (5,6); but the frequency of lymphoma carrying animals was also dependent upon the severity of the primary infection. This lymphoma is transplantable, but the number of tumor carrying animals was significantly larger after transplantation into mice which had suffered from severe malarial infection than after transplantation into normal mice. Furthermore, the latent period was shorter, and more mice succumbed in the first group than in the latter. The liver which was never found to be involved in cases of spontaneously developing lymphomas in untreated mice, showed tumor development in more than a third of all instances in mice previously exposed to malarial infection.

These observations support the assumption that the LRS may be prone to malignant change on the basis of an intense malarial in-

fection in the presence of some virus. The role of the "conditioned" LRS is demonstrated by the fact that the histological picture of the solid lymphoma is relatively uniform, though obviously different types of virus particles are present in the tumor. These particles resemble the infectious agents of common murine leukemias with the exception of their size (4,6); the histological picture of the solid lymphoma however, differs from that of common murine leukemias. Whereas the genetic constitution of the host appears to play an important role in the type of leukemia induced by the same virus (7), different virus agents may cause a more uniform pathological picture due to a more uniform stimulation of predisposed cells, as indicated by our results.

Although some papers dealt with the role of the LRS during malaria infection (8, 9, 10, 11, 12, 13, 14), the changes occurring of the cellular level, responsible for malignant transformation are largely unknown. In a recent paper Sengers et al., (15) reported a disturbed immunological responsiveness during *P. berghei* infection. Infected mice showed delayed and inhibited skin heterograft rejection and were unable to form antibodies to heterologous erythrocytes. One might assume that due to a disturbed immunological responsiveness, immunologically controlled murine oncogenic and leukogenic viruses which are common in conventional mice (16), are activated. However, no increase in number of virus particles could be observed during the acute phase of malarial infection.

The general assumption is that cell lesions, or malignant transformation of cells by viruses depend on a certain interaction between the viral genome and the host genome (17). Malarial infection could initiate this interaction (5). Not only were virus-like particles found to persist in cultured cells up to 5 months (12), but an EB virus nucleic acid homologue could also be demonstrated in a line of Burkitt tumor cells which was assumed to be "virus-free" (19).

Another hypothesis is that the leukemia virus does not induce leukemia by direct action on host cells, but rather that leukemia results when a host immune response produces secondary damage in virus infected cells (20). This could explain the absence of large numbers of virus particles during the acute phase of malarial infection and the long latency period between malaria infection and the occurrence of lymphomas. This would also imply that the damaged cells to which virus particles almost exclusively were found to be attached are not altered by the virus; but virus production is initiated by an immunological damage of these cells.

The reasons for the preferred lymphoma growth in the liver of animals previously infected with *P. berghei* are unknown; but could be explained by the following mechanism. The sinusoidal endothelium (s.e.) belongs to the RES and reacts more or less strongly dependent on the degree of parasitaemia (8, 21).

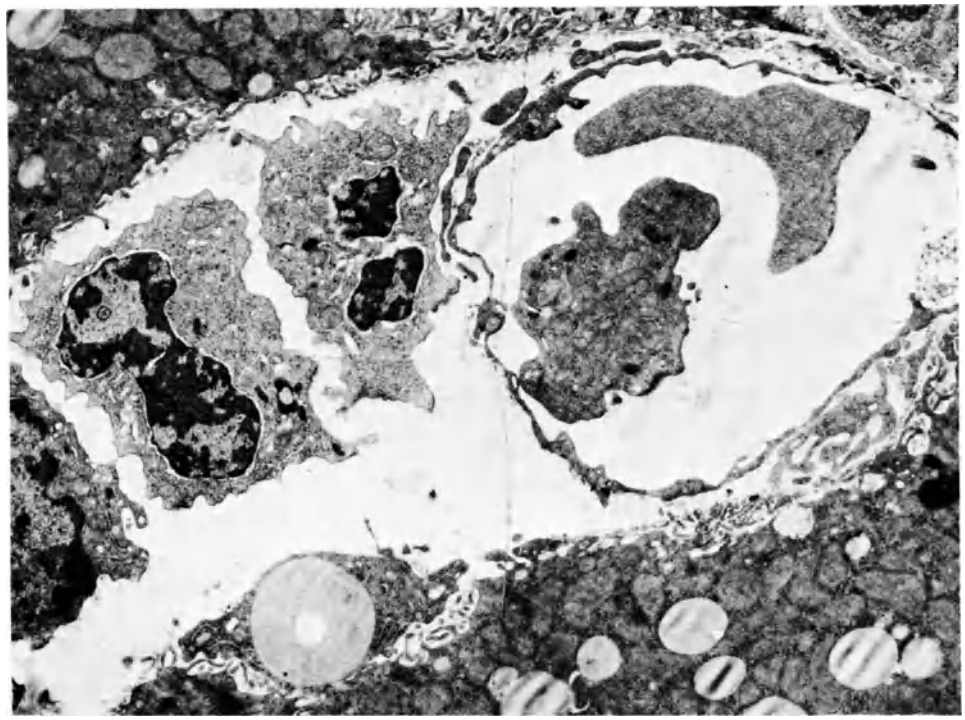

Fig. 2. Liver, SWISS mouse, 7 days after infection with P. berghei. Exfoliation of the sinusoidal endothelium, and accumulation of cells of the histio- monocytic seris in the intracellular space.

In case of severe malarial infection, the s.e. exfoliates and will be partly replaced by blood borne cells of the reticulohistocytic series (22). Some of these cells leave the sinusoids for the space of Disse and finally accumulate in the periportal areas (fig. 2). Since these cells derive from lymphatic organs,they therefore can be "conditioned" for malignant transformation.

Although the cellular basis of malignant transformation remained obscure, the results obtained point to a relationship between malaria infection and the incidence of a virus induced aleukemic malignant lymphoma.

SUMMARY

The occurrance of an aleukemic lymphoma, resembling histologically Burkitt's lymphoma, in Swiss mice immune to the malaria parasite *P. berghei* has been described. The percentage of mice carrying aleukemic lymphomas was strongly dependent on the severity

of the primary infection with *P. berghei*. Virus particles of different sizes and features were seen on electron micrographs of lymphoma tissue, particularly during the early stages of tumor growth.

Lymphomas transplanted by cell free extracts of lymphoma tissue into normal mice never involved the liver; however, when transplanted into mice immune to *P. berghei*, the liver was involved in 33% of the tumor bearing animals.

The question is discussed as to whether malaria infection may "condition" the normal LRS and RES to malignant change in the presence of some virus or viruses, or whether different virus agents may cause a more uniform pathohistological picture due to a more uniform stimulation of predisposed cells.

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PATHOMECHANISM OF INCREASED RES ACTIVITY IN RATS BEARING YOSHIDA SARCOMA

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It was observed in rats bearing subcutaneous Yoshida sarcoma that the growth of the tumors is associated with considerable splenomegaly. Considering that in the opinion of Andreini et al. /1/ this splenomegaly of the tumor bearing anímals is due to an immunological reaction and this view has recently been confirmed by other authors /2, 3/, it seemed worth while to investigate the immunological relations of the Yoshida sarcoma. The question seemed all the more promising as Scheiforth et al. /4/ have reported that their rats, bearing subcutaneous Yoshida sarcoma, did not demonstrate an isoimmune response and that they could not observe either a tumor inhibitory or transplantation reaction. Circulating antibodies were not present either in the serum of the tumor bearing animals.

The aim of our investigations was as follows:

1. Does the histological picture of the enlarged spleen exhibit immunologic histological patterns?

2. How does the tumor growth influence the activity of the reticuloendothelial system and what role does that splenomegaly play in the development of enhanced RES activity?

3. Does the previously implanted and then surgically removed tumor tissue give an immune response in the host and does it induce the inhibition of the growth of a tumor transplant implanted later into the organism of the host?

Method

The experiments were performed on female albino rats

weighing 160-180 g of the R-Amsterdam strain kept on a standard diet. The transplantation of the tumors was carried out by means of a trocar, a piece of tumor tissue /about 50 mg/ was inoculated and the transplantation was successful in 100 per cent. The histological studies of the spleen and the determination of the RES activity were performed on the 15th day after tumor implantation.

For histological studies the spleen was fixed in formaldehyde and stained with haematoxylin-eosin or methyl-green-pyronin.

The RES activity was determined by the method of Biozzi et al. /5/. For the examinations Pelikan India ink /C 11/1431a, Gunther Wagner, Hannover/ was injected intravenously /16 mg/100 g body weight/ and the phagocyte index /k/ determined. The animals were killed by decapitation in the 13th minute and small pieces of the spleen and liver were used for histological studies to determine the India ink content.

In other 30 rats the role of splenomegaly in inducing enhanced RES activity of the tumor bearing animals was examined. Ten controls did not receive any pretreatment, in 10 other animals splenectomy was performed and 10 were submitted to a sham-operation 2 days before the implantation of the tumor. The RES activity was also determined in these animals on the 15th day of the tumor growth.

Considering that according to our previous experiments a successful removal of a subcutaneous Yoshida tumor is on the 10 to 12th day not possible without recurrence of the original tumor, the transplantation into the tail was attempted as suggested by Andervont /6/ and later Thunold /7/. These authors applied this technique for the transplantation of tumors in mice and were able to remove the tumor surgically by the section of the tail at a definite time of the tumor growth. In this manner Thunold /7/ could induce tumor resistance to Ehrlich ascites carcinoma in 20 of 45 mice. For these investigations 50 rats were used with a corresponding number /fifty/ of controls. Following the transplantation the cutting of the tail was carried out on the 21st day.

Results and discussion

On the 15^{th} day of the tumor growth when its average weight was 18.7 g the spleen of the tumor bearing animals was considerably enlarged. The weight of the spleen calculated for 100 g body weight was 135 mg in the controls and 318 in the tumor bearing rats. The difference was significant /$p < 0.001$/. According to the histological studies the hypertrophy of the Malpighi follicles and increase in number

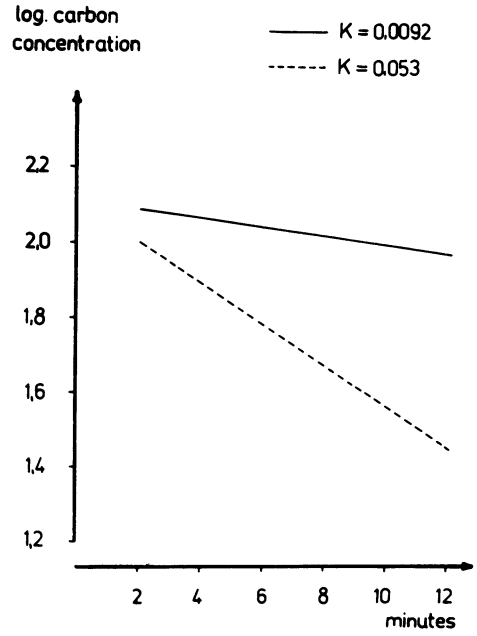

Fig. 1. Carbon clearance examinations: ——— controls
------ tumor bearing animals
$p < 0.001$.

of the pyroninophil cells is clearly visible.

The investigations showed that on the 15th day of the tumor development the RES activity was considerably enhanced. Whereas, the "k" value of the controls was 0.0092 that of the tumor bearing rats 0.055 /Fig. 1./. According to the mathematical analysis, the difference is very significant.

Considering the results of Biozzi et al. /5/ that the degree of the RES activity depends mainly on the weight of the liver and spleen, it seemed worth while to investigate what is the role of splenomegaly in the development of enhanced RES activity in the tumor bearing animals. We have demonstrated that the enhancement of the RES activity was independent of the presence of the spleen as it also developed in the group of animals which had undergone splenectomy 2 days before the tumor transplantation /Fig. 2./.

The histological studies confirmed the results obtained with the India ink clearance method. Whereas, in the histological sections showing the India ink filling in the

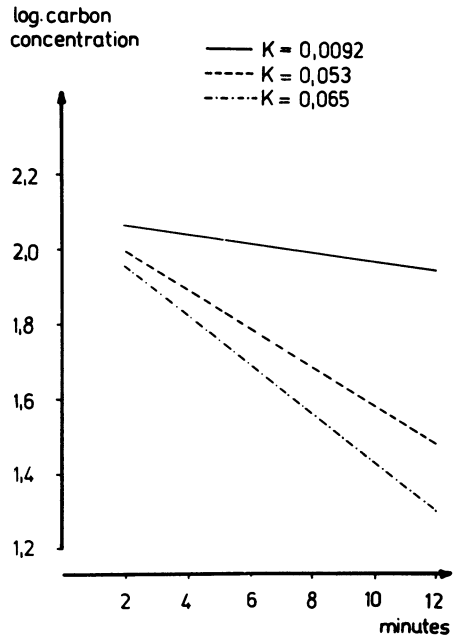

Fig. 2. Carbon clearance examinations:

——— controls
– – – – sham-operated tumor bearing animals
$p < 0.001$
– · – · splenectomised tumor bearing animals
$p < 0.001$

spleen of the tumor bearing animals and the controls there seemed to be no difference, in the liver of the tumor bearing animals an appreciable enhancement of the India ink phagocytosis ensued /Fig. 3, 4./. Our histological studies showed that for the increased RES activity in tumor bearing animals, the considerable enhanced phagocytic function of Kupffer cells is responsible.

We consider that the enhanced RES activity in the rats with subcutaneous Yoshida sarcoma is due to an immunological reaction. This is confirmed by the fact that in our experiments by tumor grafts into the tail, complete tumor resistance could be induced.

After the transplantation of the tumor into the tail on the 6th to 10th days following the inoculation a palpable, flat tumor could be observed. In the majority of the animals,

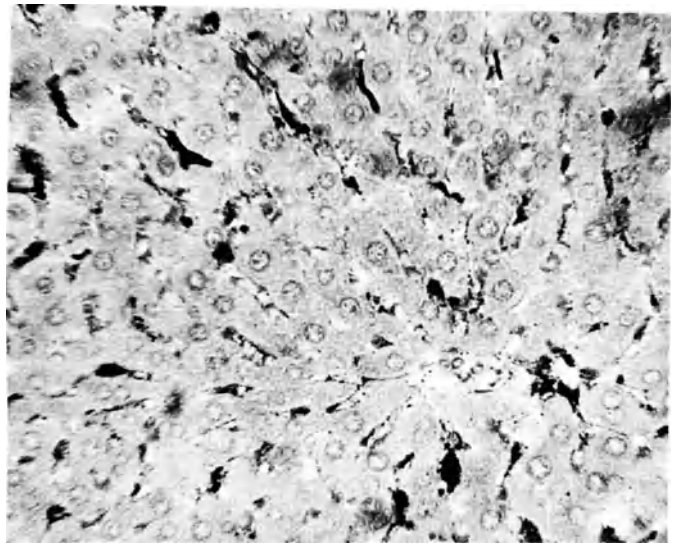

Fig. 3. Liver of a control rat 13 minutes following the intravenous injection of India ink. H.E. x 240.

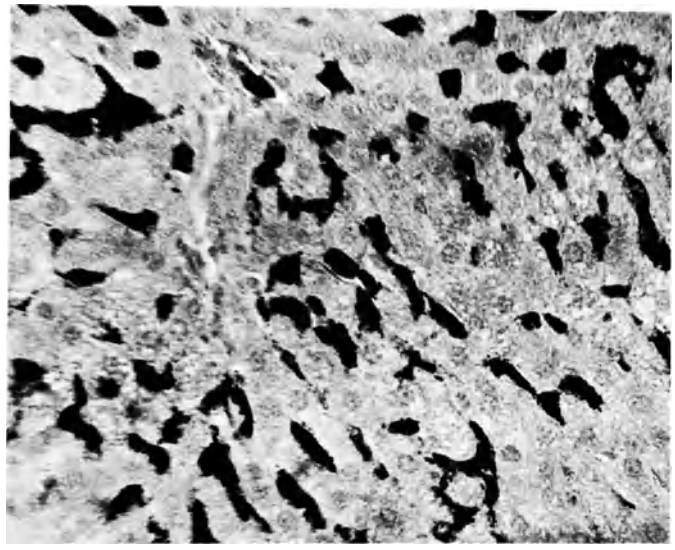

Fig. 4. Liver of a tumor bearing rat 13 minutes following the intravenous injection of India ink. H.E. x 240.

/70 %/ the growth of the tumor hardly increased in the following period and after 3 weeks gradually regressed. In a smaller percentage of the animals /25 %/, the tail transplant showed a progressive development, and owing to the extensive tumor growth their tails had to be cut off on the 21st day. Finally in about 5 per cent of the rats the animals were killed between the 21st and 30th days because of the metastases formed in the abdominal cavity.

On the 30th day following the tumor transplantation into the tail the second tumor inoculation was performed. In all the controls the tumor developed progressively, while in the animals in which precedingly a tumor had been grafted the tumor growth did not ensue in a single case, not even when the transplantation was repeated several times.

Summary

According to our experiments, the subcutaneous Yoshida sarcoma evokes immune response in rats. This statement is based on the following observations:

1. The spleen of animals with tumor significantly increases and the histological picture of the enlarged spleen corresponds to immunological reaction.

2. According to our experiments by carbon clearance method in tumor bearing animals the RES activity significantly increases. The enhancement of the RES activity is not connected with the splenomegaly associated with the growth of the tumor, as it also developed in splenectomized tumor bearing animals. Our histological studies showed that for the increased RES activity the considerable enhanced phagocytic function of the Kupffer cells is responsible.

3. By tumor grafts into the tail regression of the tail tumor and complete tumor-resistance could be induced.

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ACTIVE IMMUNOTHERAPY BY B.C.G.

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Immunotherapy is a new method of killing malignant cells in the treatment of cancer. It is well known,from the rejection of incompatible grafts, that membrane antigens can excite an immune reaction capable of killing cells carrying these antigens. It is also known that these immune reactions can sometimes fail to kill the cells that have raised the reaction and, indeed, work quite to the contrary by protecting the cells : this is the enhancement phenomenon (1).

When the malignant cells and the immunologically competent cells are allogeneic,the immunotherapy is, in fact, the application of the immunogenetics of incompatible grafts ; if the malignant cells are syngeneic to their host, but carry specific neoantigens induced by chemical carcinogens or viral carcinogens, an immune reaction can be made by the host's own lymphoid tissue (2,3,4). As a general rule, when they act on grafted tumours or on clinically detectable tumours in man, these immune reactions - when they exist - are incapable of destroying the whole population of tumour cells, but it is conceivable that if this tumour cell population was reduced in size, and the immune reactions increased, the final result of the conflict between the tumour proliferation and cell destruction by the immune reactions, could lead to cure. We have studied this hypothesis, using murine leukaemia as an experimental model, and in

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man in the treatment of acute lymphoblastic leukaemia.

1. EXPERIMENTAL FINDINGS IN MICE

The first step in mice was clearly to demonstrate that the syngeneic leukaemic cells, which were going to be used as a target for active immunotherapy, carried specific antigens from their malignant transformation, for this would be indispensable for the immune reactions that we wish to stimulate. This fact is now well established in mice, and tumour specific antigens are present in viral induced leukaemias such as the E♂G2 leukaemia or the Charlotte Friend leukaemia, or in leukaemias induced by chemical carcinogens, such as the L 1210 (5,6).

The second step is to develop techniques capable of stimulating the immune reactions against the membrane antigens - that is to say, for non-specific active immunotherapy, agents capable of stimulating the reactions against an incompatible graft. This step has been surmounted some time ago, since B.C.G. and Corynebacterium parvum, as well as certain of their extracts, were shown to be able to accelerate the rejection of incompatible tumour grafts (7).

The third step consists of knowing if the immune reactions that are stimulated in this way against the membrane antigens and capable of assuring the rejection of incompatible tumours in a more constant fashion, and in a shorter time, than in controls, could equally assure the rejection of a syngeneic tumour or leukaemia, only relying on the specific membrane antigens from the malignant transformation.

This step is essential to transfer the experimental results across to the treatment of man : in 1967, J.-L. Amiel showed, that the E♂G2 leukaemia, a leukaemia in C57Bl/6 mice, induced by the Gross virus, and carrying antigens of the Gross system, grafted in F1(DBA/2 x C57Bl/6) hybrid recipients, could be cured by B.C.G. when it was applied at the optimal moment and at the optimal dose. This was found to be 15 days before the injection of the leukaemic cells, and cures were obtained only if the number of malignant cells did not exceed 10^4. If the number reached 10^5, immunotherapy could only cause a prolongation of life, and it had no effect when the number of cells was 5×10^5.

When B.C.G. is given in a single injection, at the optimum moment, that is, fifteen days before the graft, one cannot increase the effect of immunotherapy for a given number of malignant cells by repeating injections

of B.C.G. or associating it with other forms of active non-specific immunotherapy, for example, Corynebacterium parvum, or adding active specific immunotherapy, by injecting AKR allogeneic leukaemic cells (8). On the other hand, when the B.C.G. is given outside the optimum period, and delayed until the first day after the injection of the syngeneic malignant cells, Mathé et al (9) have shown that they are able to improve the immunotherapeutic effect by adding active specific immunotherapy, using irradiated L 1210 cells, to the non-specific effect from the B.C.G. By injecting L 1210 leukaemia subcutaneously, the evolution of the grafted cells in an animal given immunotherapy 24 hours after as the malignant cells (10), can be studied. At first, the leukaemia cells proliferate, then either the tumour regresses and the animal is cured, or the animal dies without regression of the tumour, or the growth enters a plateau phase for some days, or several weeks, when there is an equilibrium between the number of cells killed and the number of cells produced (9). These studies have not shown any difference in the sensitivity of cells to active immunotherapy, according to whether they are in cyclic division or out of cycle (Go). Using this experimental model, Mathé and his colleagues have confirmed the principal idea that there is a maximal number of cells, above which value no immunotherapeutic effects can be obtained. In their model this number is 10^5, and this is the same order as has previously been shown by Amiel.

The fourth step in this argument is eventually to know how active immunotherapy from B.C.G. is best used in a scheme for a treatment of a compatible leukaemia - that is, to know the best sequence of cytostatic chemotherapy and active immunotherapy to obtain an optimal result. Amiel and Berardet (11) have demonstrated that an immunosuppressive cytostatic chemotherapy given at the same time or after non-specific active immunotherapy from B.C.G., completely abolishes this active immunotherapeutic effect. It is even clear that the immunosuppression obtained by the sequential treatment of B.C.G. followed by an immunosuppressive cytostatic is greater, and particularly more long-lasting, than that obtained when the immunosuppressive cytostatic is given alone (11).

On the other hand, when the graft of malignant cells contains more cells than the maximum number that active immunotherapy can control, it is still possible to obtain cures by using the sequence of cytostatic chemotherapy followed by active immunotherapy (12). A graft of syngeneic EOG2 leukaemia, containing 10^5 cells was established, without treatment, for four days ; then chemotherapy, using methyl hydrazine, was given from the fourth

to the ninth day using a dose which, alone, only prolonged the life of these animals. Non-specific active immunotherapy, using B.C.G. or Corynebacterium parvum, was started on the tenth day, and led to a very high percentage of cures.

The results of experiments made with non-specific active immunotherapy with B.C.G. show a good correlation to experiments using active specific immunotherapy with irradiated syngeneic cells or allogeneic cells having the same specific antigens as the living malignant cells that are to be killed (9). The substances that have been shown to be effective in non-specific active immunotherapy are equally effective as stimulants of other forms of immune reactions. For example, against heterospecific red cells (13). The first hypothesis is that the effect of B.C.G. is certainly a non-specific active immunotherapeutic effect, but it is positive that no clear correlation has been established between the stimulatory effect of B.C.G. on the reticulo-endothelial system and its active immunotherapeutic effect (14). The precise mechanism of the action of B.C.G. on syngeneic leukaemias remains largely unknown.

Some information can be obtained from these experiments to help to plan the trials of active immunotherapy in man : the method is efficient, its essential limitation is to be effective only on a small number of malignant cells, its essential advantage is that it can produce definite cures in compatible leukaemia. To be most effective, it should not be followed or accompanied by immunosuppressive cytostatic chemotherapy, but it can be used after this treatment. To increase effectiveness of non-specific active immunotherapy from B.C.G. by giving active specific immunotherapy, using cells carrying the same membrane antigens as those to be killed, could be very important, when one is outside the optimal conditions for B.C.G.'s action, and in particular when it is given after a graft of malignant cells (9).

2. ACTIVE IMMUNOTHERAPY TRIALS IN MAN

The existence of specific antigens from malignant transformation has not been so clearly demonstrated on human leukaemic cells as on murine leukaemic cells. Doré et al (4), and Yoshida and Imai (15) have produced evidence which, at the very least, are strong presumptions for the existence of these antigens. Acute lymphoblastic leukaemia presents many good reasons for it to be the subject of the first trials of active immunotherapy. It is a generalised disease which cannot be cured by surgery or radiotherapy ; the disease is sensitive to chemo-

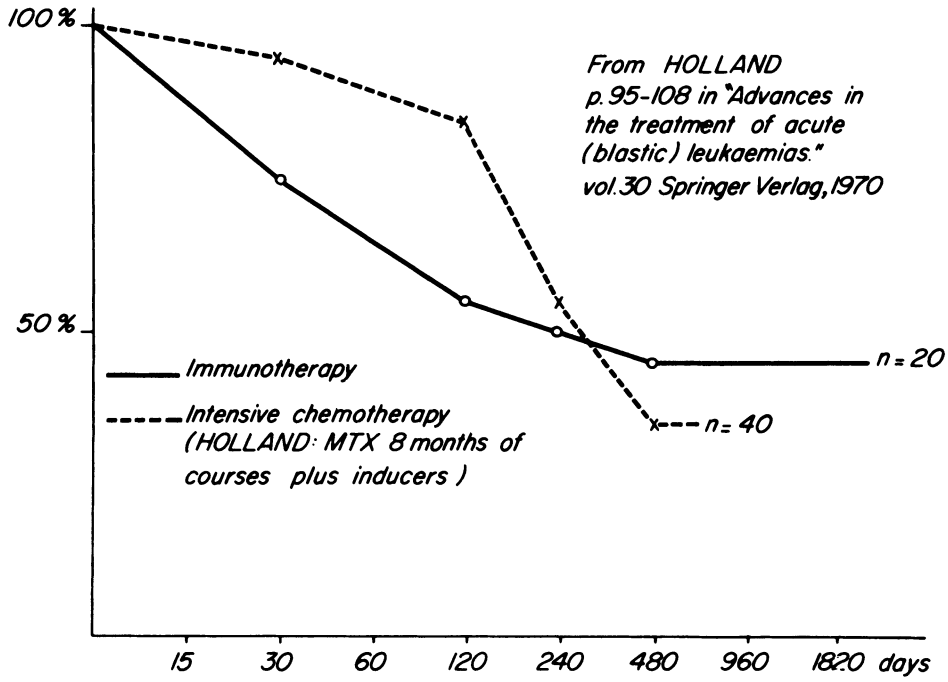
therapy, and complete remissions can be obtained - that is, there is a considerable reduction of the total cell population but, apart from a few exceptions, these remissions never become long-lasting cures. In many aspects acute lymphoblastic leukaemia is close to the experimental models in mice, where active immunotherapy has been shown to be the most effective.

In our first trial (16, 17, 18), started in 1964, 30 patients, whose ages varied from 3 to 50 years, were treated in this way ; they received after remission induction, a sequential complementary cell-reducing chemotherapy (one drug at a time) combined with intrathecal administration of methotrexate and meningeal radiotherapy. Four groups were formed randomly. In the first, 10 control patients did not receive any further treatment after complementary chemotherapy was stopped. In the second, 8 patients were treated by B.C.G. and received, on every fourth day and then on every eight day, twenty cutaneous scratches, each 5cm long, and arranged in a square. 2ml of a suspension containing 75mg/ml of living bacteria were put into the scarified area. In the third group, 5 patients received each week, both intradermally and subcutaneously, 4×10^7 leukaemic cells, which had been obtained from a pool of allogeneic donors suffering from acute lymphoblastic leukaemia. These cells had been preserved at -70°C in DMSO. For the first six injections, the cells were treated with a 4 p. 100 formol solution to inactivate any possible virus, and, for remaining injections, they were irradiated with 4,000 rads *in vitro*. In the final group, 7 patients were given both forms of immunotherapy.

Each of the 10 patients left without treatment after chemotherapy was stopped, relapsed. The average duration of the remission was 66 days, the median lying between 70th and 77th day. The limits were 30 and 130 days. At the 130th day, only 9 of the 20 patients given immunotherapy had relapsed, and the difference between these two groups is highly significant ($\chi^2 = 6.18$, $p = 0.02$). Later 4 other patients relapsed. An examination of the relapses gives rise to the following ideas : a) the majority of them appeared to be early : 9 before the 100th day, 5 before the 30th day, which is comparable with the experimental observations in mice, and suggests that the number of tumour cells left after the chemotherapy was greater than the maximum number that could be controlled by active immunotherapy ; b) 4 of them were late in onset. One occured at the 210th day, another at the 315th day, and another at the 950th day (which is comparable with those exceptional relapses we had noted in our experiments with mice). The fourth, which occurred at the 324th

day, was in an infant in whom the BCG-treatment had been stopped 44 days previously (because of a severe and intractable phlyctenular keratoconjunctivitis which had required the treatment to be stopped several times). This last patient, when considering the possibility of late relapses, answers the question as to whether immunotherapy should be continued indefinitely. Seven patients are still in remission more than two years after chemotherapy has been terminated ; in five it is more than 3 years, and in two it is more than four years.

The actuarial curves demonstrate differences between the groups submitted to immunotherapy and the control group. They indicate that, although the median for the remission duration in our patients given immunotherapy is of the same order as those given intensive chemotherapy (19), the shape of the curves of these two groups is quite different. That of the patients given immunotherapy tends to straighten out and continue as a plateau (Fig.1).



Actuarial curves of the duration of complete remission

——— Active immunotherapy after stopping chemotherapy

----- Intensive chemotherapy (from Holland, 1970)

There were no significant difference between the group given B.C.G. (5 relapses out of 8 patients ; treatment had been stopped in one), that given leukaemic cells alone (3 relapses in 5 patients) and the group given both these forms of immunotherapy (5 relapses in 7 patients).

The remarkable results and the failures of this trial require further discussion. With regard to the successful results, it is important to remember that the patients who underwent immunotherapy had been highly selected. That is, they all received prolonged chemotherapy, with little effect. It is possible that these patients belonged to a special category and perhaps were destined for long spontaneous remissions. However, it should be noted that the control individuals relapsed very rapidly, confirming the inefficiency of chemotherapy. In regard to the design of the trial, it is the chemotherapy that should be reviewed, in terms of the poor choice of certain drugs which rarely act against acute lymphoblastic leukaemia, and in terms of sequential administration of only a drug at a time.

In a following protocol, whose results are only preliminary and have not been published, the complementary cytoreductive preimmunotherapy treatment was as intensive as possible. The chemotherapeutic drugs were given two at a time in four sequences of chemotherapy; chemotherapy using two drugs was given intrathecally and radiotherapy was given to the central nervous system. A splenectomy was carried out in patients with splenomegaly, and any enlarged lymph nodes were treated by radiotherapy. At the end of this cytoreductive therapy, immunotherapy was commenced. One third of the patients received B.C.G., Corynebacterium parvum and leukaemic cells, one third the same treatment combined with a monthly injection of vincristine, and one third the same treatment combined with a continuous treatment with adamantadine, an antiviral agent (20). The preliminary results are very encouraging. In the group of patients given this therapy in the first visible phase of their leukaemia, only one relapse has occured in those who receive immunotherapy alone (the longest period is only 16 months). Relapses have occured in patients given a combination of immunotherapy and chemotherapy and in those given immunotherapy and adamantadine. These results confirm our experimental findings that 1) chemotherapy administered at the same time as immunotherapy inhibits its effects (11); 2) adamantadine is an immunosuppressive agent (21).

A fourth protocol of therapy is starting to be used in which the action of poly-IC, a new adjuvant, on residual disease is to be studied, because we have shown that

poly-IC has an action on leukaemia in the visible phase providing the number of leukaemic cells are still fairly low (22).

In conclusion, the experience that we have obtained with our clinical trials shows good agreement with those in the experimental studies. Active immunotherapy, in particular, active immunotherapy with B.C.G., has been shown to be effective in the control of acute lymphoblastic leukaemia in man, as it is in various forms of murine leukaemia. In man, as in mice, the essential factor appears to be a reduction, as far as possible, of the number of malignant cells by chemotherapy, before giving active immunotherapy ; in man, as in mice, the addition of any immunosuppressive treatment during the active immunotherapy phase can clearly detract from the beneficial result. It remains to hope that in man, as in mice, the long remissions under active immunotherapy will, in fact, turn out to be cures.

It is possible that a better understanding of the mechanism of action of non-specific active immunotherapy, the development of new immuno-stimulant substances, such as poly-IC, ought to be able to improve the results obtained by active immunotherapy in the treatment of human leukaemia, but the progress depends just as much on better forms of cytoreductive chemotherapy given systematically, and intrathecally, before attempting immunotherapy.

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AUTHOR INDEX

(Underscored numbers indicate complete paper in this volume)

SUBJECT INDEX