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HAEMOLYTIC ACTIVITY OF HUMAN BLOOD MONOCYTES

LYSIS OF HUMAN ERYTHROCYTES TREATED WITH ANTI-A SERUM

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SUMMARY

Purified monocytes and lymphocytes from peripheral blood of healthy human donors were tested *in vitro* for cytotoxicity against blood group A erythrocytes (RBC) treated with a human hyperimmune anti-A serum. Haemolysis was quantitated by the release of radioactivity from RBC pre-labelled with ^{51}Cr-chromate.

The antiserum contained high titre antibodies which agglutinated A-RBC. After separation of serum on a Sephadex G-200 column the IgG containing fraction agglutinated A-RBC and precipitated blood group A substance. No or only weak antibody activity was detected in the IgA- and IgM-containing fractions.

Purified monocytes added to a 100-fold excess of RBC in the presence of 0·1% antiserum induced some haemolysis. It was calculated that one monocyte was able to lyse 2–3 RBC within 18 hr incubation. In contrast, lymphocyte suspensions containing more than 97% small lymphocytes had no or only weak haemolytic activity at a lymphocyte-RBC ratio of 25 : 1. The effector cells of the monocyte fraction adhered to glass and were eliminated by incubation with carbonyl iron. Phagocytosis by monocytes of antibody-treated RBC was frequently observed. Loading of monocytes by treatment with heat-killed *Candida albicans* or carbonyl iron particles suppressed their haemolytic action. Cytotoxicity was impaired after treatment of monocytes with 10^{-4} M sodium iodo acetate. After separation of serum on Sephadex G-200 column all monocyte induced haemolytic activity was found in the IgG containing fraction. It is assumed that haemolysis is induced by the interaction of monocytes with an IgG anti-A antibody of the antiserum.

INTRODUCTION

In human immune haemolytic diseases destruction of red blood cells (RBC) coated with antibody and/or complement is usually effectuated by cells of the monocyte phagocytic

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system particularly in the spleen and the liver (for references, see Pirofsky, 1969; Dacie, 1970). Complement-fixing antibodies may lyse RBC in the circulation probably without the aid of effector cells. The mechanisms of haemolysis *in vivo* is not known in detail. Erythrophagocytosis may be a main way of destruction but other mechanisms have been proposed. Although erythrophagocytosis by polymorphonuclear neutrophils (PMNs) has been observed in immune haemolytic diseases monocytic phagocytes are assumed to be the main effector cells of lysis.

Blood monocytes have morphological and functional properties in common with tissue macrophages (Huber, Douglas & Fudenberg, 1969; Braunsteiner & Schmalzl, 1970; Hirsch & Fedorko, 1970). When cultivated *in vitro* they develop into typical macrophages (Bennett & Cohn, 1966). Studies in animals using ^{3}H-thymidine-labelled cells may indicate that blood monocytes which are derived from precursor cells in the bone marrow are the mobile form of tissue macrophages (Volkmann, 1966; van Furth & Cohn, 1968; van Furth & Diesselhoff-Den Dulk, 1970). Consequently, *in vitro* studies of the haemolytic effect of human blood monocytes on human erythrocytes treated with antibody and/or complement may clarify some mechanisms of haemolysis in human immune haemolytic diseases.

In a previous report a ^{51}Cr-chromate method for *in vitro* quantitation of cell-mediated lysis of antibody-treated human red cells was described (Holm, 1972). Human blood leucocytes or peritoneal macrophages damaged autologous or allogenic erythrocytes treated with various isoantisera. Lysis was not caused by purified blood lymphocytes. This paper describes pronounced lytic activity of purified human blood monocytes on A erythrocytes in the presence of a hyperimmune human isoantiserum containing anti-A antibodies mainly of IgG class. Monocytes lysed an excess of antibody-treated RBC. Erythrophagocytosis was shown to be one mechanism of lysis. Purified human blood lymphocytes did not lyse RBC treated with this particular antiserum.

MATERIALS AND METHODS

Anti-A serum

Antiserum was obtained from a healthy O Rh(+) donor hyperimmunized with hog blood group A substance. One batch of this serum was used in all experiments. Pooled serum from healthy AB Rh(+) donors served as control serum. Sera were inactivated at 56°C for 1 hour and stored at −20°C in small portions.

Medium

Parker 199 supplemented with 2 mM glutamine, antibiotics (100 units of penicillin and 100 μg streptomycin per ml) and 10% heat-inactivated foetal bovine serum (Microbiological Associates, Bethesda, Md., U.S.A.) was used in all experiments.

Hanks's solution buffered with an equal volume of isotonic Tris, pH 7·4 was used for cell washings (=HT).

Monocytes

Monocytes were prepared from human peripheral blood with a modification of the method by Bennett & Cohn (1966). Leucocytes obtained by gelatine sedimentation (Coulson & Chalmers, 1964) of 100–150 ml heparinized blood were washed once by centrifugation at 100 *g*. Leucocytes were suspended in 3·2 ml of heparinized HT and mixed in a siliconized

tube with 10·8 ml 35% bovine serum albumin (KABI, Stockholm, Sweden) to give a final concentration of 27% albumin. The mixture was divided into two siliconized graded tubes. On the top of the gradient 3·4 ml of HT was added carefully to give a total volume of 10 ml. The tubes were centrifugated at 400 *g* for 40 min at room temperature. The white cell layer at the interface was collected and washed twice in HT with 1% gelatine by centrifugation at 100 *g* for 10 min.

In some experiments monocytes were further purified by absorption to plastic tissue culture flasks (Falcon Plastics, Los Angeles, U.S.A.) as described in the text.

Lymphocytes

Lymphocytes were purified from the gelatine supernatant (or from the monocyte fraction) by incubation with carbonyl iron and elimination of phagocytic cells with a magnet (Greenwalt, Gajewski & McKenna, 1962). The cells were cleared from red blood cells (RBC) by centrifugation in a Ficoll-Isopaque gradient (FIP) (Böyum, 1968).

Differential count and morphology

The viability of cells in suspension was tested after staining of dead cells with trypan blue. A preliminary differential count was made on leucocyte suspensions in Türk's stain. The definite differential count was made by counting 300 cells on coded smear preparations stained with May-Grünwald and Giemsa.

Phagocytosis

The ability of leucocytes to ingest heat-killed *Candida albicans* or A-RBC coated with anti-A antibodies was determined as described previously (Holm, 1972).

Determination of haemolysis

RBC from heparinized blood were washed three times in HT. $20–30 \times 10^6$ RBC were incubated in 0·5 ml HT with 200 μCi sodium ^{51}Cr-chromate (Radiochemical Centre, Amersham, England) at 37°C. After 30 min 1% trypsin was added and the mixture was incubated for another 30 min. The cells were then washed three times and counted in a Bürker chamber.

10^5 ^{51}Cr-RBC were mixed with effector cells and antiserum in a total volume of 1·5 ml medium in 15 × 110-mm sterile conical plastic tubes (Heger Plastics, Stallarholmen, Sweden). The mixture was incubated at 37°C in a humid atmosphere of air with 5% CO_2. After incubation the tubes were centrifuged and 0·5 ml of cell free supernatant was pipetted off for counting of radioactivity. The radioactivity of the cell free supernatant expressed as a percentage of the total radioactivity of the tube is used as a measure of haemolysis (for further details, see Holm, 1972).

Haemagglutination

The haemagglutinating titres were determined by incubation of A-RBC with two-fold dilutions of the antiserum as previously described (Holm, 1972).

Complement-mediated lysis

Fresh guinea-pig serum was used as a source of complement after absorption with trypsinized human red cells of blood group A. 10^5 ^{51}Cr-labelled RBC were suspended in

conical plastic tubes with antiserum and absorbed guinea-pig serum diluted 1:20 in a total volume of 0·6 ml. After 60 min incubation at 37°C the tubes were centrifuged. The release of radioactivity to the supernatant was determined.

RESULTS

Characterization of the anti-A serum

The antiserum agglutinated A Rh(+) RBC at the dilution 1:1000. The haemagglutinating titre of trypsinized A-RBC was 1:8000. After separation of the antiserum on a Sephadex G-200 column, the main haemagglutinating activity was found in the IgG containing

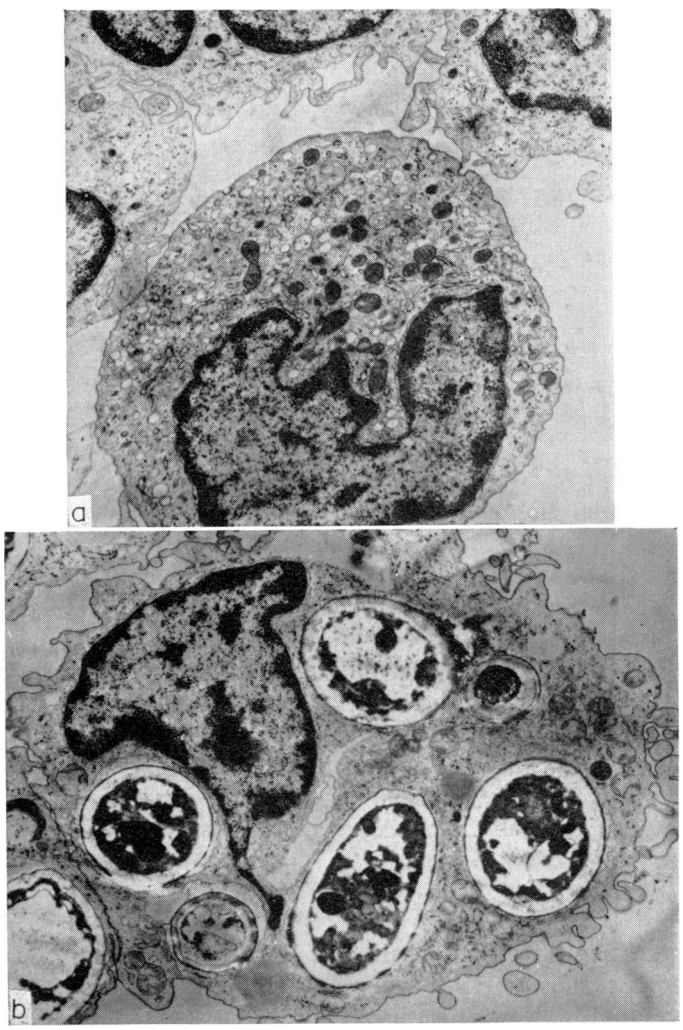

FIG. 1. Electron-micrograph of purified monocytes after incubation with or without heat-killed *Candida albicans* for 2 hr. Glutaraldehyde-osmium fixation (Biberfeld, 1971). (Magnification, ×17,600.)

fraction. Low titre haemagglutinins were present in the IgA and IgM containing fractions. The IgG fraction specifically precipitated purified blood group A substance. Precipitating anti-A antibodies were not detected in the IgM fraction. Trypsinized RBC were completely lysed by 0·4% anti-A serum in the presence of complement. 0·05% antiserum had no effect while 50% lysis was induced by 0·15% serum.

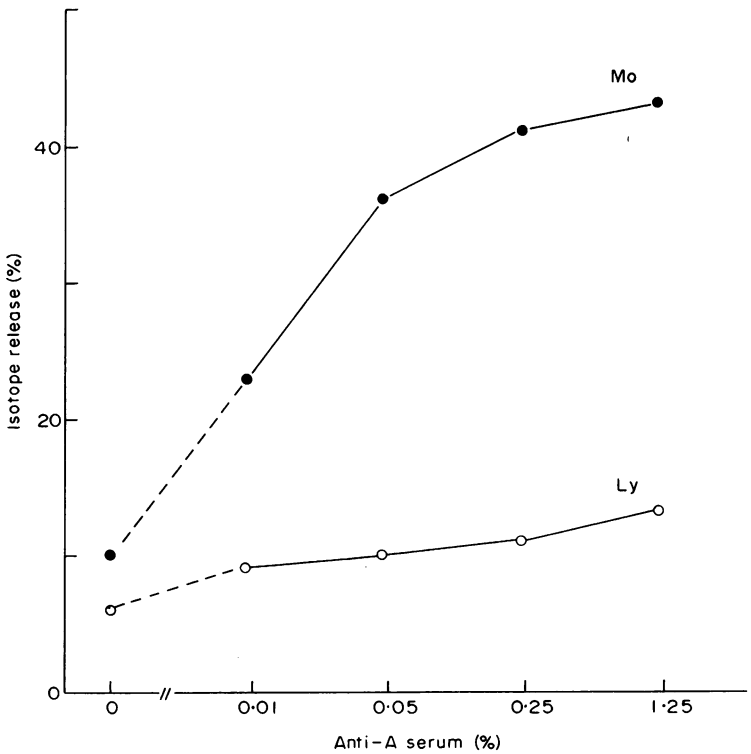

FIG. 2. Comparison between haemolytic effects of lymphocytes and monocytes from the same donor at different concentrations of antiserum. Leucocyte donor, blood group O Rh(+).

Mo: Monocytes prepared with albumin gradient. Ratio 10 : 1.
Ly: Lymphocytes purified by the iron method. Ratio 25 : 1.

Differential counts (%):	monocytes	lymphocytes	PMN
Mo:	98	2	0
Ly:	0	94	6

Time of incubation, 48 hr.

Isolation of monocytes

The albumin gradient separation usually yielded 10–20 × 10^6 monocytes from 100–150 ml blood. The monocytes were contaminated with varying amounts of lymphocytes (usually 5–20%). A few polymorphonuclear neutrophils (PMN) and RBC were also present. Preparations containing more than 10% PMNs were discarded. After incubation of the monocyte preparation for 3–18 hr in tissue culture flasks more than 95% of the surface adhering cells were monocytes (Fig. 6). Almost all cells of such preparations had electron microscopical

characteristics of monocytes and were phagocytic as revealed by their ability to engulf heat-killed *Candida albicans* (Fig. 1).

Haemolytic effector cells

Albumin gradient separated monocytes lysed RBC in the presence of low concentration (0·01%) of heat-inactivated anti-A serum (Fig. 2). Lysis did not increase significantly with anti-A concentrations higher than 0·05%. Purified lymphocytes from the same donor had only weak haemolytic effect in the presence of high concentrations of antiserum. Antibody-treated A-RBC were lysed by autologous (Fig. 3) as well as isologous monocytes (Fig. 2). In the presence of 0·1% anti-A serum one monocyte per 100 RBC was sufficient for lysis

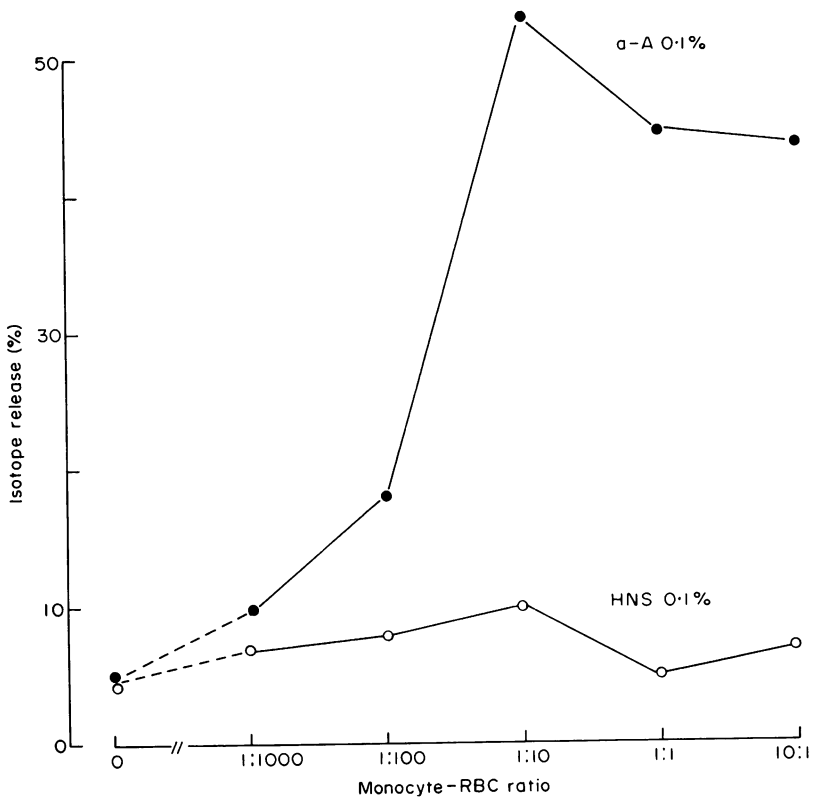

FIG. 3. Haemolytic effect of low concentrations of monocytes. Monocytes and RBC from the same donor, blood group A Rh(+). Differential counts: 92% monocytes, 3% lymphocytes, 5% PMN. Time of incubation, 20 hr.

of some RBC (Fig. 3). No further haemolysis was observed when the monocyte-RBC ratio was increased from 1:10–10:1. On the basis of these observations the experimental conditions were standardized as follows: 10^5 monocytes were incubated with 10^5 RBC in the presence of 0·1% anti-A serum for 18–20 hr.

The haemolytic efficiency of blood monocytes is illustrated in the experiment of Fig. 4

where monocytes were incubated with increasing concentrations of ^{51}Cr-labelled RBC. 50% lysis was induced by monocytes added at the monocyte-RBC ratio of about 1:5, suggesting that one monocyte may lyse 2–3 RBC within 18 hr of incubation.

To exclude that albumin gradient fractionated leucocytes contained a population of lymphocytes with haemolytic activity, experiments exemplified in Fig. 5 were performed. Albumin separated leucocytes lost their haemolytic activity almost completely after elimination of phagocytic cells by the iron ingestion method. The iron-treated fraction which contained mainly small lymphocytes had low haemolytic activity similar to that of the total lymphocyte population purified from blood by iron-FIP.

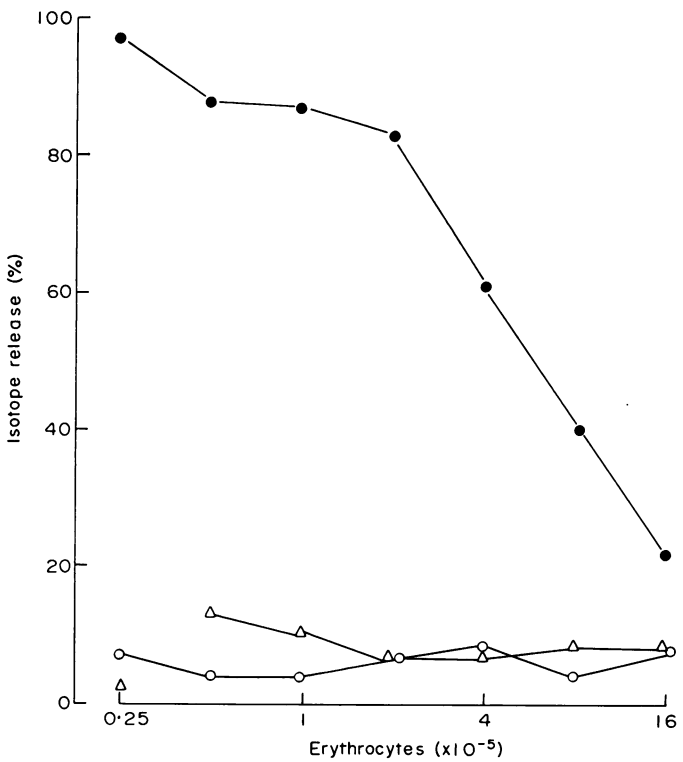

FIG. 4. Haemolytic efficiency of monocytes. 10^5 monocytes were incubated with the number of A red cells indicated on the abscissa in the presence of 0·1% anti-A serum (filled circles) or 0·1% control AB serum (open circles). Open triangles, RBC incubated with anti-A serum alone. Time of incubation, 18 hr.

Monocytes were purified further from albumin gradient separated leucocytes by absorption to plastic surface (Fig. 6). Highly purified monocytes (fraction A1) were most cytotoxic, while lymphocyte rich preparations had no or only weak activity (fractions B1 and B2).

Kinetics of monocyte-mediated lysis

Under the experimental conditions described in Materials and Methods monocyte induced release of radioactivity usually started after a latency period of 30–60 min (Fig. 7).

It then increased rapidly during the next 3 hr. Later, there was no or only slight further lysis. It should be noted, however, that in some experiments lysis continued for 24–48 hr although the rate of lysis was slower than that of the initial phase.

The lag phase of lysis was probably due to slow sedimentation of the suspended cells. The reaction was speeded up when the cells were sedimented by slow centrifugation (80 *g* for 3 min) prior to incubation (Fig. 8). Lysis was then completed within 1–2 hr incubation.

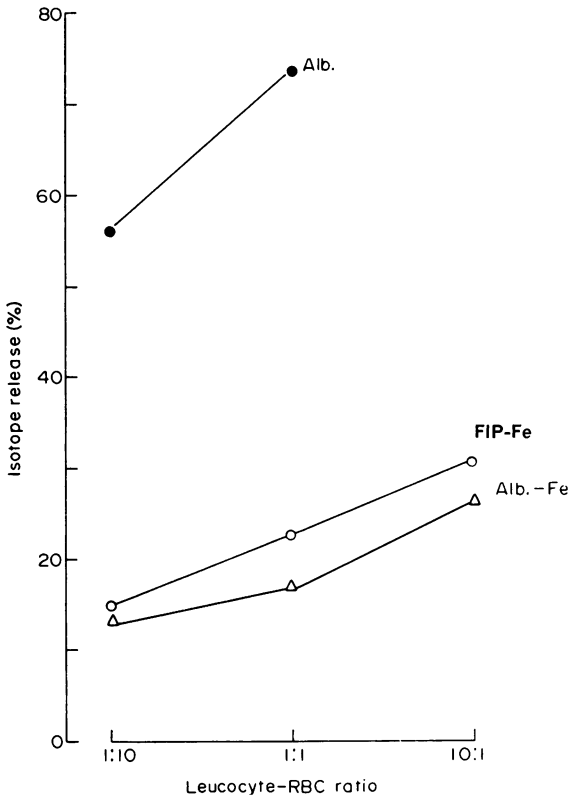

FIG. 5. Comparison between haemolytic effects of monocytes and lymphocytes from the same donor. Leucocyte donor, blood group O Rh(+).

Alb: Crude monocyte fraction obtained by albumin sedimentation.
Alb-Fe: Elimination of phagocytosing cells in the Alb-fraction by ingestion of iron.
FIB-Fe: Lymphocytes purified by Ficoll-Isopaque and iron.

Differential counts (%):	monocytes	lymphocytes	PMN
Alb:	84	8	8
Alb-Fe:	1	97	2
FIP-Fe:	1	99	0

0·1% Anti-A serum present. Time of incubation, 18 hr. Spontaneous isotope release, 7%.

Phagocytosis

Phagocytosis of antiserum-treated RBC by monocytes was frequently observed. After pre-incubation of monocytes with heat-killed *Candida albicans* they partly lost their haemolytic

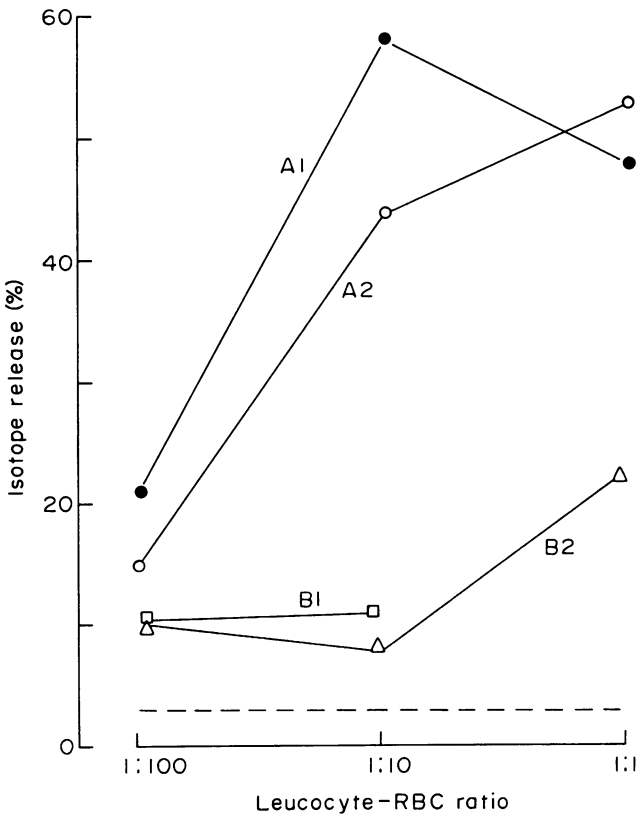

FIG. 6. Haemolytic effects of leucocyte fractions. Leucocyte donor, blood group AB(+). The monocyte fraction obtained after centrifugation on albumin gradient was incubated in medium on tissue culture flasks for 3 hr at 37°C. After two washes, fresh medium was added to the adherent cells and incubation was continued overnight (fraction A). Non-adherent cells were incubated on new flasks overnight (fraction B). The medium was collected and the flasks were washed once. Adherent cells were detached by treatment with 0·25% trypsin for 5 min at room temperature (fractions A1 and B1, respectively). Non-adherent cells recovered from medium and washings were treated simultaneously with trypsin (fractions A2 and B2 respectively).

Differential counts (%)	monocytes	lymphocytes	PMN
Fraction A1	98	1	1
A2	86	9	5
B1		no smears	
B2	7	93	0

0·1% Anti-A serum present during incubation. Time of incubation, 18 hr. Dotted line: Spontaneous isotope release.

activity (Table 1). Morphological examination of such preparations revealed monocytes loaded with fungi (Fig. 1). Monocytes which had been treated with carbonyl iron particles did not lyse antibody-treated RBC (Table 1).

Sodium iodo acetate, an inhibitor of glycolysis and phagocytosis (Cohn, 1970), suppressed haemolysis when added to the incubation mixture at 10^{-3}–10^{-4} M concentrations (Fig. 9).

The viability of monocytes checked with trypan blue was not impaired by this treatment.

Induction of haemolysis by serum fractions

The anti-A serum was fractionated on a Sephadex G-200 column. The IgM and IgG containing peaks were collected as well as the intermediate IgA containing fraction and the albumin containing peak. The fractions were tested at concentrations which approximately corresponded to 1:500 dilution of the original serum. Under these conditions haemolysis was induced by monocytes only in the presence of the IgG fraction (Table 2).

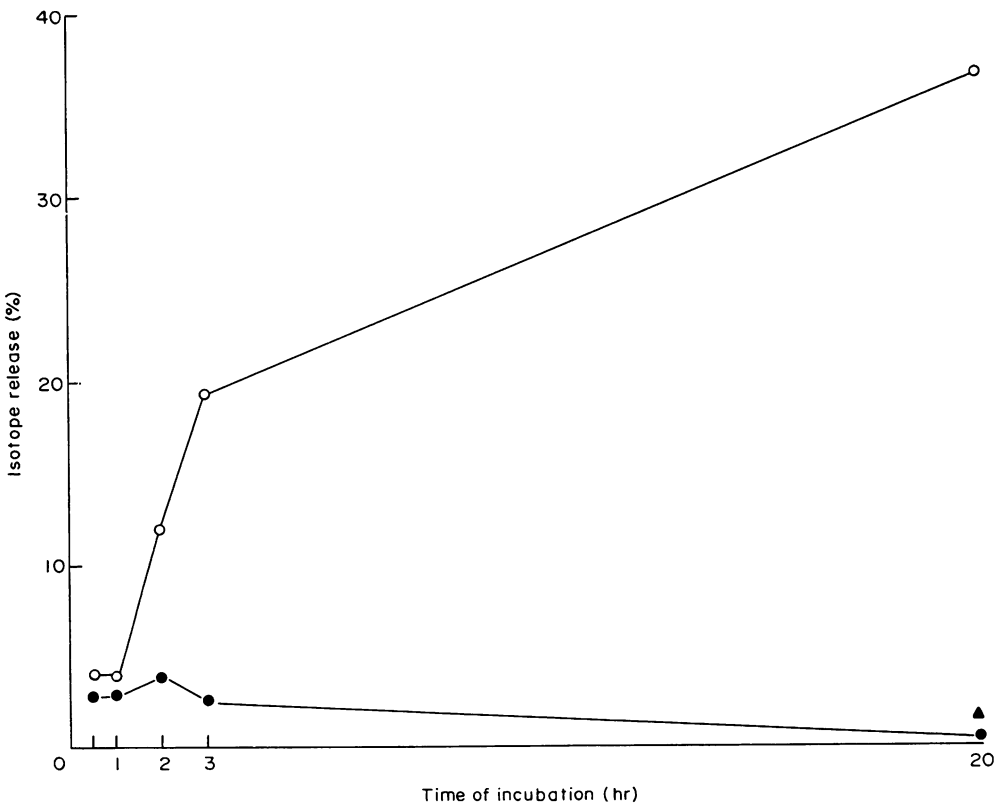

FIG. 7. The effect of time of incubation. Monocyte donor, blood group O Rh(+). Differential count: 90% monocytes, 6% lymphocytes, 4% PMN. Monocyte-RBC ratio, 1:1. ○, Monocytes+0·1% anti-A serum; ●. monocytes only; ▲, anti-A serum only.

DISCUSSION

Blood monocytes are here defined as cells with the morphology of monocytes under light and electron microscope which are phagocytic and which can attach to glass. The morphology of monocytes on light microscopical level is often indistinguishable from that of lymphocytes (Hirsch & Fedorko, 1970). Although differential counts were made on coded preparations, it is likely that some monocytic cells were erroneously classified. However, when highly purified monocytes were incubated with heat-killed *Candida albicans* almost all cells exhibited

phagocytosis, while phagocytic cells were rarely detected in purified lymphocyte preparations.

Cytotoxic monocytes could be separated from inactive cells having the morphology of lymphocytes by 3-hr absorption to plastic surface (Fig. 5). By prolonged incubation some monocytes detached from substrate but retained their lytic activity (fraction A2, Fig. 5).

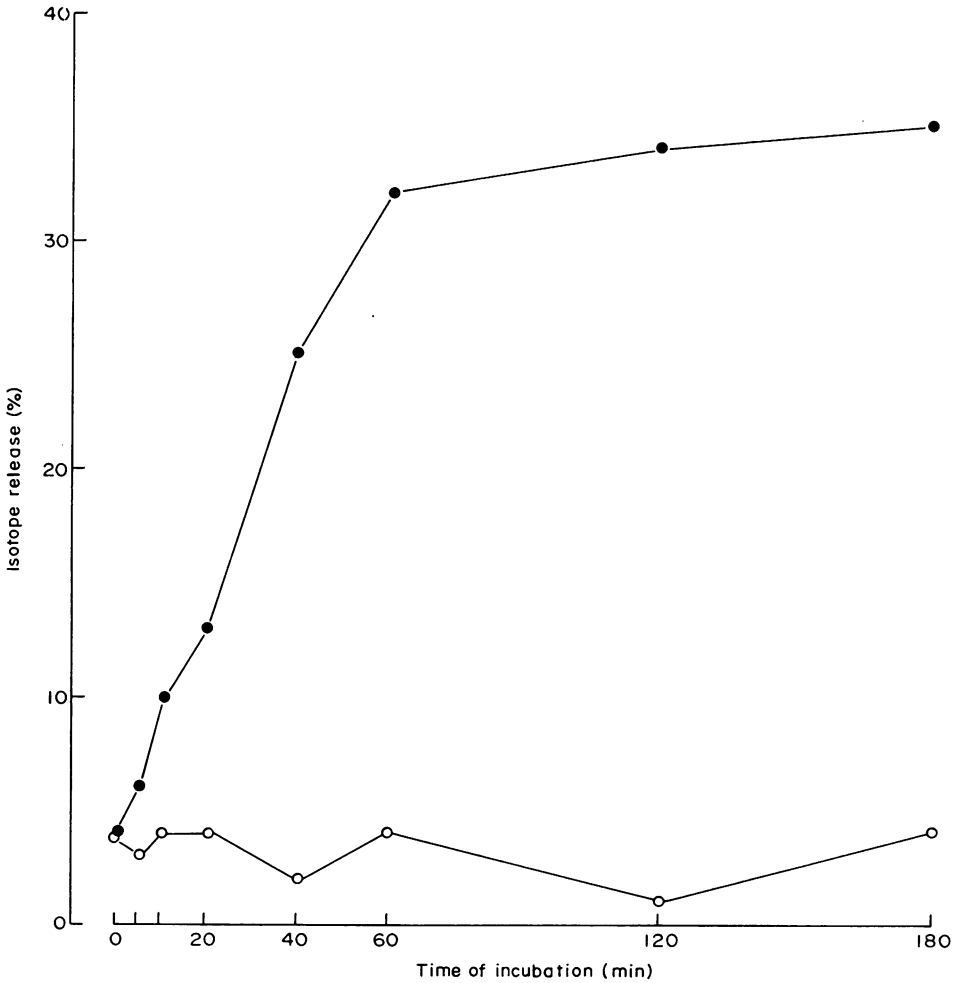

FIG. 8. Rapid haemolytic effect of monocytes after centrifugation of the monocyte-RBC mixture (80 *g*, 3 min). Monocyte donor, blood group O Rh(+). Differential count: 22% monocytes, 73% lymphocytes and 5% PMN. Monocyte-RBC ratio, 1:1. ●, Monocytes+0·1% anti-A serum; ○, monocytes only.

Furthermore, when phagocyting cells were eliminated from albumin separated leucocytes by iron ingestion the preparation lost its haemolytic activity.

The data show that blood mononuclear phagocytes are effector cells in the lytic reaction with A red cells treated with this particular antiserum. The presence of total complement was not required for lysis. Under optimal experimental conditions one monocyte may lyse

two to three erythrocytes and the rapid phase of the reaction was finished within 1-hr incubation. In contrast, purified lymphocytes consistently failed to lyse target cells. The weak haemolysis which was sometimes induced by purified lymphocytes added at high lymphocyte-RBC ratio may be an effect of contaminating monocytes.

Lysis of human red cells induced by nylon purified human lymphocytes in the presence of anti-A serum from the same donor as used in this investigation was studied by Hinz & Chickosky (1972). Using trypsinized and ^{51}Cr-labelled RBC they found that red cells were

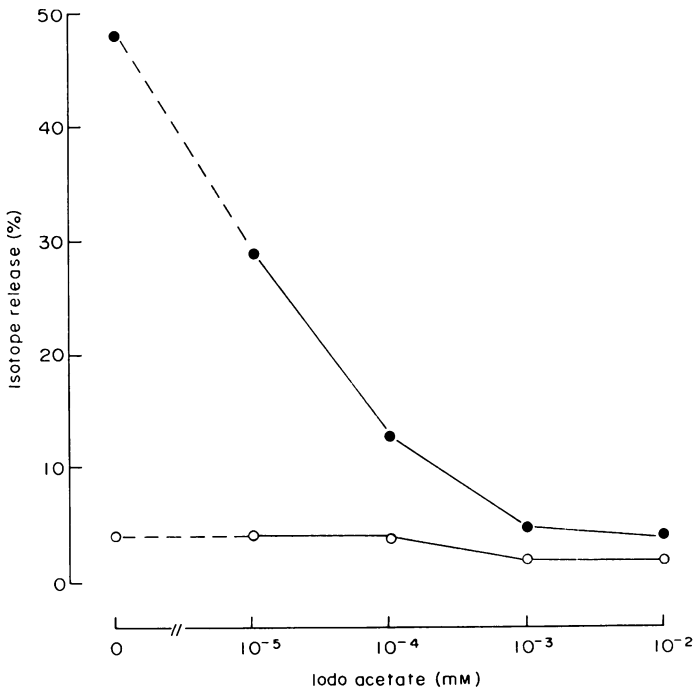

FIG. 9. Inhibition of monocyte mediated haemolysis by sodium iodo acetate added to the incubation mixture. The monocyte-RBC suspensions were spun at 80 *g* for 3 min prior to incubation. Time of incubation, 3 hr. Monocyte donor, blood group AB Rh(+). Differential count: 43% monocytes, 55% lymphocytes and 2% PMN. ●, Monocytes + 0·1% anti-A serum; ○, monocytes only. Viability of monocytes (exclusion of trypan blue) after incubation with iodo acetate for 3 hr:

Iodo acetate (mM)	–	0·01	0·1	1	10
Dead cells (%)	2	1	1	2	60

lysed by a twenty-fold excess of lymphocytes during 48-hr incubation in the presence of 2·5% heat-inactivated antiserum. Crude leucocytes induced rapid haemolysis within 3 hr. Similar effects were induced by nylon purified lymphocytes and high titre anti-D sera. They concluded that the slowly induced haemolysis was an effect of lymphocytes while neutrophils and monocytes induced rapid lysis. Similar conclusions were drawn from kinetic experiments of the lytic reaction between human leucocytes and chicken erythrocytes coated with rabbit antibody (Perlmann & Perlmann, 1970).

The present results do not conclusively exclude weak haemolytic action of lymphocytes during 24–48-hr incubation with human red cells in the presence of this particular antiserum. Also, lymphocytes may co-operate with monocytes in the lytic reaction.

TABLE 1. Inhibition of monocyte induced haemolysis by pretreatment with particles

Incubation	Isotope release (%)	
	Anti-A serum (0·1%)	HNS (0·1%)
Monocytes	63	7
Monocytes preincubated with *Candida albicans*	43	18
Monocytes preincubated with iron	14	16
No monocytes	9	6
No monocytes, 10^6 *Candida* added	8	7
No monocytes, 1 mg iron added	12	10

Monocyte donor, blood group O Rh(+).

5×10^6 Monocytes in 2 ml medium were incubated alone or with 125×10^6 heat-killed *Candida albicans* or with 0·05 g carbonyl iron powder for 1 hr at 37°C on a rocking table. The cells were washed three times in siliconized tubes.

Differential counts: 78% monocytes, 15% lymphocytes, 7% PMN.

Ratio monocytes-RBC, 1:1.

Time of incubation, 20 hr.

HNS, normal human AB serum.

To facilitate haemolysis the erythrocytes were labilized by pretreatment with trypsin (Holm, 1972). Trypsinization, however, was not necessary for monocyte induced lysis. Untreated RBC were damaged by monocytes in the presence of this anti-A serum in concentrations which were about ten times higher than those required for lysis of trypsinized RBC (unpublished observations).

Phagocytosis of antiserum-treated RBC by monocytes was frequently observed and may be one of the mechanisms of haemolysis in this system. When erythrophagocytosis was impaired by loading the monocytes with particles (*Candida* or carbonyl iron), their haemolytic activity was inhibited. In these experiments it can not be excluded that phagocytosis

TABLE 2. Haemolysis by fractionated antiserum

	Isotope release (%)	
	Monocytes present	No monocytes
Sephadex G-200 fractions:		
IgM containing fraction, 0·2%	5	6
IgA containing fraction, 0·2%	5	5
IgG containing fraction, 0·2%	20	5
Albumin containing fraction, 0·2%	4	8
Anti-A serum 0·1%	25	7

Monocyte-RBC ratio, 1:1. Time of incubation, 2 hr.

was impaired by toxic effects of the particles. Moreover, particles which attached to the monocyte surface may block the contact to opsonized RBC. Monocytes treated with 10^{-4} M sodium iodo acetate lost their lytic activity. This substance blocks phagocytosis (Cohn, 1970) but also suppresses non-phagocytic lysis of chicken red cells or tissue culture cells mediated by activated lymphocytes in close contact (Perlmann & Holm, 1969). Hence, the experiments do not exclude that non-phagocytic mechanisms such as contact mediated lysis contribute (Lo Buglio, Cotran & Jandl, 1967). Experiments are in progress to further evaluate these possibilities.

Human monocytes possess receptors for human IgG of subclass 1 and 3 which can bind IgG-coated RBC (Abramson *et al.*, 1970; Huber *et al.*, 1971). The function of the IgG receptors is probably to facilitate phagocytosis by attachment of opsonized RBC. Monocyte induced lysis of RBC was effectuated by antibodies of the IgG fraction of the anti-A serum. In preliminary experiments monocyte mediated lysis of RBC treated with this antiserum was inhibited by 10 μg/ml of pooled human IgG (data to be published). The results support the assumption that haemolysis in this particular system is effectuated by mechanisms related to phagocytosis and induced by IgG antibodies of the antiserum.

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