Interaction of serum components with the cytotoxic action of "Entamoeba histolytica"

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Summary

Native normal human serum is capable of inhibiting the cytotoxic action of *Entamoeba histolytica* against K562 tissue culture target cells assessed by a ^{51}Cr-release test. It is suggested that a part of the inhibitory activity on amoebae's cytotoxic action is represented by the complement system which is known to lyze trophozoites by activation of the alternative pathway. For the rest of the serum's inhibitory activity on amoebic cytotoxic action molecule(s) is (are) responsible which act(s) independently of Mg^{2+} and Ca^{2+}. The serum components have to act before the trophozoites have come in contact with the target cells. The opsonization of trophozoites with antiamoebic antibodies led to an inhibition of amoebae's cytotoxic action (ACA) in a dose-dependent manner. The inhibitory components of normal human serum and antiamoebic antibodies potentiated each other in their capacity to inhibit ACA. It is suggested that opsonization of amoebae with C3b via its metastable binding site leads to redistribution phenomena on the amoebae's surface similar to the effects observed with antiamoebic antibodies, both events leading to inhibition of ACA.

Key words: *Entamoeba histolytica;* cytotoxic action; serum components; antiamoebic antibodies.

Introduction

The influence of humoral defense mechanisms on amoebae can take place by direct interaction with the parasite or by a modulation of its cytotoxic action.

A direct influence on the parasite has been shown already by Stemberger (1978), Huldt et al. (1979), Ortiz-Ortiz et al. (1978), and Kollaritsch et al. (1985), inasmuch as the trophozoites apparently are activators of the alternative pathway of complement. Trophozoites are lyzed in the absence of antibodies by fixation of the third component of complement and activation of the late complement components. There are several published data regarding the action of antibodies on *Entamoeba histolytica:* An antibody dependent cellular cytotoxicity (ADCC) is not active against live amoebae, it is only effective in vitro, when erythrocytes are artificially coated with amoebic extracts, that is, when the target cells possess a rigid surface (Stemberger, 1978). If antibodies are added together with complement the lytic effect of the latter is not enhanced (Stemberger, 1978). An explanation for this observation was given by Trissl et al. (1977), Aust-Kettis and Sundquist (1978), Calderon and Tovar-Gallegos (1980), Aust-Kettis and Utter (1984), and others. Antibodies apparently are partially internalized or induce redistribution events of surface components towards the uroid (Biagi et al., 1966). A cap is formed which is finally released and thus shedding of immune complexes takes place. Coating the trophozoites with antibodies did not substantially enhance the action of the complement cascade in the sense of activation via the classical pathway (Stemberger, 1978). Nevertheless immune complexes shed from the amoebae's surface proved to be activators of the classical pathway (Hudler et al., 1983). On the other hand, the influence of antibodies, complement and/or other factors of human serum on the cytotoxic action of trophozoites are comparatively not so well established.

In this paper we present data regarding the modulation of the amoebae's cytotoxic action (ACA) with humoral factors and antibodies directed against amoebic antigens.

Materials and Methods

Amoebae

Trophozoites of the strain SFL3 were monoxenically cultured in medium TYI-S-33 (Diamond, 1968a, 1968b; Diamond et al., 1980) modified as follows:

Ingredients	Amounts/l
Biosate peptone (Beckton, Dickinson and Co.)	20.00 g
Yeast extract	20.00 g
Glucose (water-free)	10.00 g
NaCl	10.00 g
$K_2HPO_4 \times 3\ H_2O$	1.31 g
KH_2PO_4 (water-free)	0.60 g
Cystein	1.00 g
Ascorbic acid	0.20 g
Ferric ammonium citrate	0.0228 g

These compounds were dissolved in 800 ml aqua bidestillata, adjusted to pH 6.8 with NaOH and filled up to a final volume of 870 ml with aqua bidest. This solution was degassed for 1 h at room temperature. Afterwards 30 ml of vitamin mixture NCTC 107 (Gibco Lab. USA) and 100 ml bovine serum (pooled normal bovine serum from slaughterhouse-blood, heat inactivated at 56 °C for 1 h and sterilized by gammaradiation) were added.

This medium was sterilized by filtration (stainless-steel-filter: SM 33001, Sartorius GmbH FRG; paper-filter: Sartobran Mini double fold paper filter, pore size 0.45/0.2 μm W.5111507H9B, Sartorius GmbH, FRG). The precise observance of the above mentioned procedures proved to be essential for successful cultivation of *E. histolytica*. For monoxenical mass-cultivation of E. histo-lytica 70 ml of the sterile medium were filled in sterile culture glass flasks (100 ml. Schott) and kept in dark at −20 °C until use. 5×10^6 trophozoites of *E. histolytica* (Strains A3, HK9 and SFL3) were inoculated to one culture flask each; 1×10^5 I.U. Na-Penicillin-G (dissolved in 1 ml sterile isotonic NaCl) and 5 ml Crithidia (grown in original TPS-1 for 3–4 days at 28 °C) were added (Diamond, 1968a).

After inoculation the cultures were incubated at 37 °C for 72 h. Trophozoites were harvested quantitatively from flasks; then washed 3 times in RPMI 1640 supplemented with 10% heat inactivated FCS, counted and tested for viability by trypan blue exclusion. (Viability ranged from 95 to 99%, counts for the 3 different strains mentioned: A3: $30 \pm 15 \times 10^6$, HK9: $25 \pm 10 \times 10^6$, SFL3: $48 \pm 13 \times 10^6$ trophozoites in one mass culture.)

Target cells

The human erythroleukaemic cell line K562 was cultured in suspension in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (RPMI/FCS; Flow Lab., Irving, U.K.). The cells were labelled with ^{51}Cr, and the microcytotoxicity assay was performed as described in detail for K and NK cells (Scheiner et al., 1980) with some modifications to test the amoebae's cytotoxic activity (ACA): 0.2 ml trophozoites (washed three times with RPMI/FCS and adjusted to 2.5×10^5/ml), 0.2 ml target cell suspension (5.10^4/ml RPMI/FCS) and 0.2 ml RPMI/FCS or antibody or human serum dilution (RPMI/FCS) were mixed and then centrifuged for 2 min at 1000 g and then incubated for various periods.

Human serum

Freshly drawn, normal human serum (NHS) was used which was negative with respect to all amoebae-specific serological tests (IIFT, IHA, ELISA, Stock et al., to be published). The complement activity was 23 CH_{50}/ml.

Antibodies

For preparation of antiamoebic antibodies, 20 ml of the serum from a patient suffering from an amoebic liver abscess was subjected to ion exchange chromatography on QAE-Sephadex A-50 (Pharmacia Fine Chemicals, Vienna, Austria). The IgG fraction was dialyzed against isotonic phosphate-buffered saline, pH 7.2 (PBS) and adjusted to 8 mg/ml. As shown with immunoelectrophoresis, this preparation proved to be pure IgG (aaIgG). For control experiments, pooled normal human Ig (16%, Hoechst Austria, Vienna, Austria) was used (nhIg).

Affinity chromatography

10^9 trophozoites were washed three times in PBS containing 2 mM phenylmethylsulfonylfluo-ride (PMSF), 10 mM EDTA and 10 mM E-aminocaproic acid (EACA) and resuspended in 5 ml of this buffer (4 °C).

The cells were homogenized by use of a Potter-Elvejhem homogenizer (0 °C). Subsequently, the homogenate was centrifuged at 1500 g for 20 min (4 °C). Thereafter, the supernatant was subjected to centrifugation at 25,000 g for 1 h at 4 °C. The pellet (plasma membranes) was resuspended in 0.5 mM $NaHCO_3$ buffer, pH 9.2 to a concentration of 2 mg protein/ml. 5 ml of this preparation were coupled to 5 ml swollen CNBr activated Sepharose 4B (Pharmacia Fine Chemicals, Vienna, Austria) according to the manufacturer's advice. 10 ml aaIgG were processed over the affinity column. Thereafter the

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gel was washed with 100 ml PBS. The amoebae specific antibodies were eluted with 0.1 M acetic acid. pH 2.7. After elution, the antibody preparation was immediately neutralized and dialyzed against several changes of PBS. Comparison of titers of this affinity-chromatographically purified aalgG with aalgG by means of ELISA (Stock et al., to be published) showed a 33-fold enrichment. Experiments with affinity purified aaIgG prepared with immobilized cytoplasmatic amoebic antigen showed that all of the capacity to inhibit ACA resided in the affinity-purified aalgG prepared with immobilized amoebic plasma membranes (25,000 g pellet, see above).

Results

Fig. 1 shows the capacity of NHS to inhibit the ACA against K562 in a dose dependent manner. An inhibition of ACA by NHS could only be shown when the serum was added to the amoebic suspension prior to the addition of target cells and subsequent copelleting. For the experiments described in this paper a preincubation period of 10 min was chosen. 50% inhibition was observed with a dilution of 1:22.5 of NHS corresponding to 1 CH_{50}/ml. NHS in a final dilution of 1:7.5 (= 3 CH_{50}/ml) exhibited almost complete inhibition of ACA against K562 target cells during the observation period (Fig. 1). When the NHS was supplemented with 10 mM EDTA, the serum only partially lost its capacity to
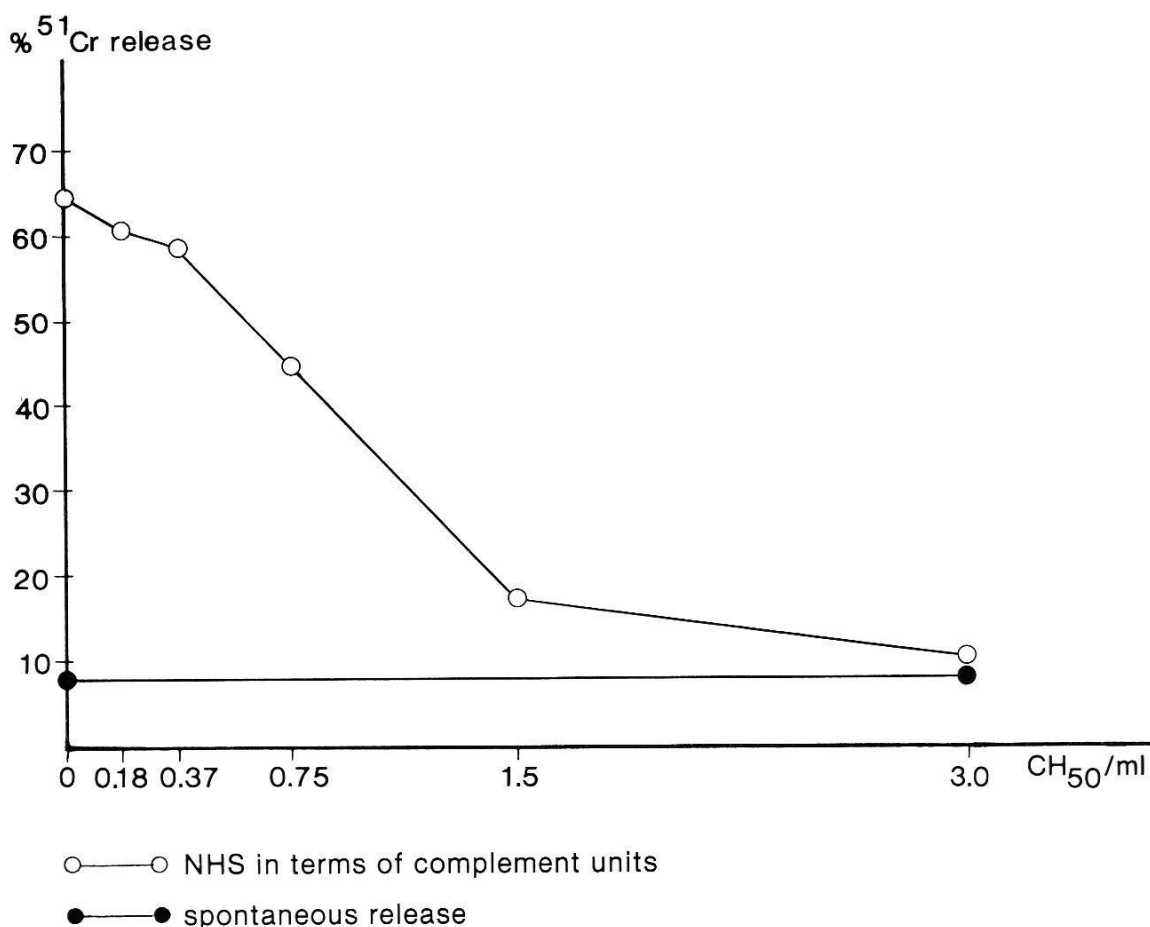

Fig. 1. Inhibitory effect of serially diluted native normal human serum on amoebae's cytotoxic activity after 30 min against ^{51}Cr-labelled target cells (effector/target cells: 5/1).
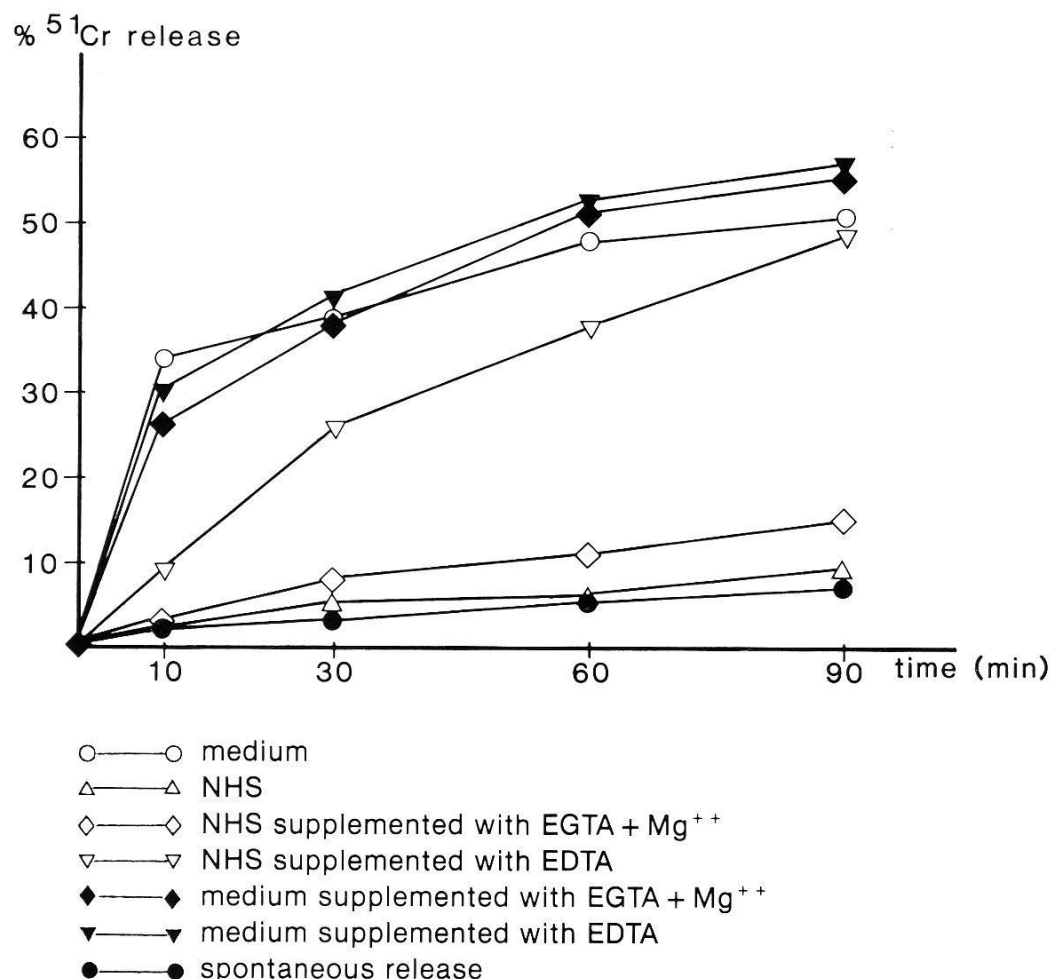
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Fig. 2. Influence of NHS supplemented with EDTA or EGTA + Mg^{++}, respectively, on NHS-dependent inhibition of amoebae's cytotoxic activity.

inhibit ACA (Fig. 2). After addition of 10 mM EGTA + 10 mM Mg^{2+} to NHS the inhibiting capacity of serum remained unchanged (Fig. 2). When the trophozoites and target cells were copelleted and held for 10 min at 37°C before addition of NHS (1:3) the inhibitory effect of NHS was completely abolished (Fig. 3a). Interestingly, the same observation was made when the preincubation of the copelleted target and effector cells took place at 4°C (Fig. 3b). However, for expression of ^{51}Cr release a temperature of 37°C proved to be necessary (Fig. 3b). Besides, antibodies directed against amoebic antigens were capable of inhibiting ACA in a dose dependent manner (Figs. 4 and 5). As can be seen, NHS + antiamoebic antibodies potentiated each other in their inhibitory capacity (Fig. 5). 2 CH$_{50}$/ml added together with all of the antibody concentrations tested (15, 60, 240 μg/ml) exerted an almost complete inhibition of ACA (Fig. 5). With 1 and 2 CH$_{50}$/ml and as little as 15 μg affinity-purified aaIgG/ml an amplified inhibition of ACA was observed (Fig. 5).
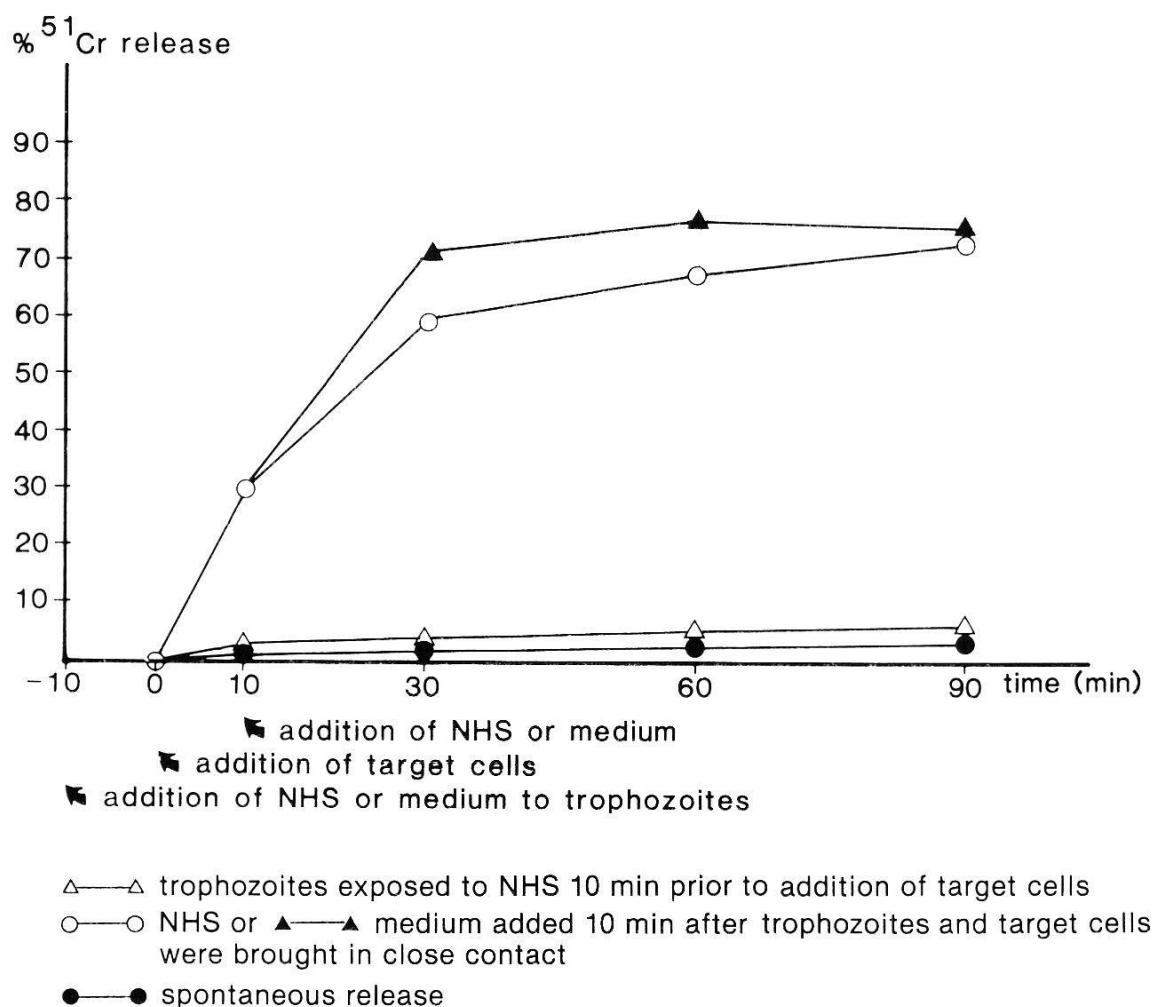
Fig. 3a. Influence of native normal human serum (NHS) on amoebae's cytotoxic activity.

Discussion

The capability of trophozoites of *Entamoeba histolytica* to lyze tissue culture target cells has been described by several authors using various experimental approaches (Knight, 1977; McCaul et al., 1977; Bos, 1979; Mattern et al., 1978; Ravdin et al., 1980; Hudler et al., 1983). Like other authors we could show that the cytotoxic activity of the trophozoites seems to correlate with their pathogenicity/virulence as assessed by the hamster liver infectivity test (Hudler et al., 1983). Conceivably, structures of the amoebae's surface are involved in the cytotoxic action, presumably by mediating contact between target and effector cell via a lectin like interaction (Kobiler and Mirelman, 1980). On the other hand, surface structures of *Entamoeba histolytica* trophozoites are obviously activators of the alternative pathway of complement (Huldt et al., 1979, Ortiz-Ortiz et al., 1978) in that amoebae were partially lyzed by native serum in the presence of EGTA + Mg^{2+}. Therefore, one should assume that abolishing of ACA by native human serum (Fig. 1) is mediated by the complement pathway in that all or most of the trophozoites are killed or at least
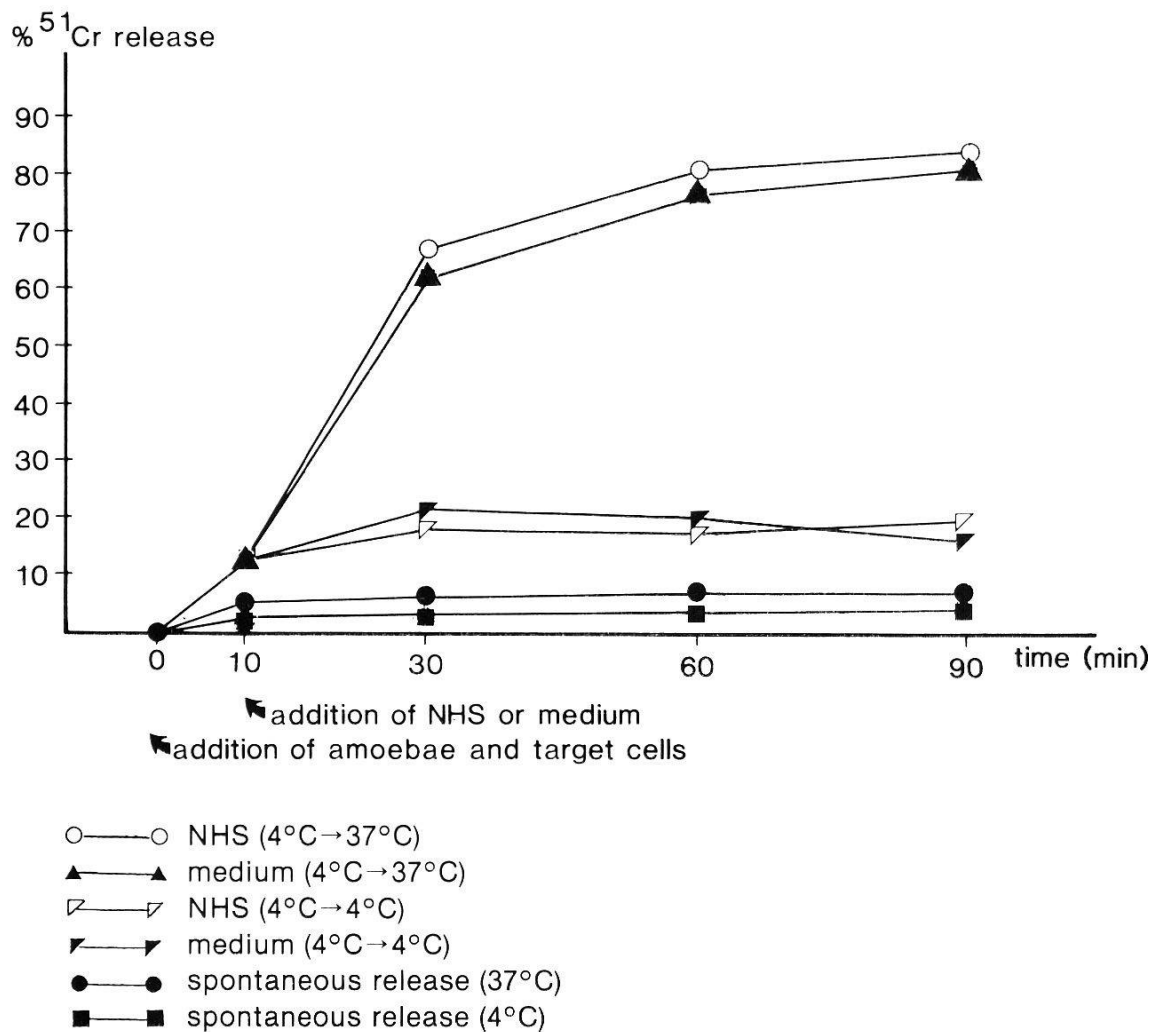
Fig. 3b. Influence of normal human serum (NHS) and temperature on amoebae's cytotoxic activity. NHS or medium was added 10 min coincubation of amoebae and target cells at 4°C. Thereafter, test tubes were kept at 4°C or brought to 37°C.

inactivated. However, addition of EDTA to native human serum only partially abolished the capacity of serum to inhibit ACA (Fig. 2). On the other hand it could be consistently shown that native serum + EDTA had no significant effect on number of trophozoites, trypan blue exclusion and release of radioactive material from labelled amoebae (Huldt et al., 1979; Kollaritsch et al., 1985) indicating that amoebae had survived in sufficient numbers to be available in that experiment. For this reason, one has to conclude that apart from the complement system there are additional serum components that act as inhibitors of ACA. These molecule(s) seemed to exert their inhibition independently of Ca^{2+} and Mg^{2+}. As yet, we cannot provide any clue to the nature of this (these) serum component(s). Possibly, these molecules act via a carbohydrate interaction, competitively inhibiting the lectin-like structures on the trophozoites surface. However, we failed to inhibit ACA by short preincubation of the amoebae with N-acetylgalactosamine, shown by Ravdin et al. (1980), to interact with the carbohydrate moiety of the amoebae's lectin (data not shown). Presumably,
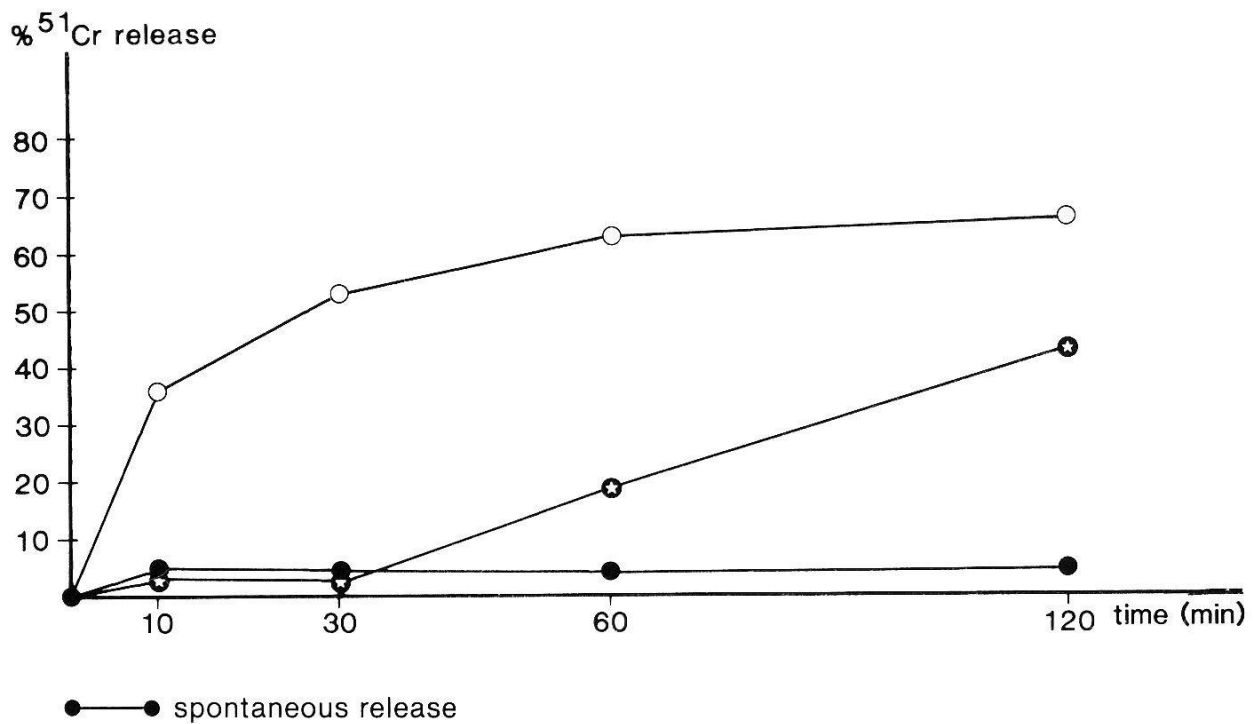
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% ^{51}Cr release

●———● spontaneous release

Fig. 4. Influence of affinity-purified human antiamoebic antibodies (aaIgG) (81.2 μg/ml) on the time course of amoebae's cytotoxic activity. Amoebae were preincubated for 10 min with aaIgG (✪—✪) or medium (○———○). Thereafter, radiolabelled K562 were added.

% lysis

□———□ medium instead of NHS
▲———▲ NHS corresponding 0.5 CH$_{50}$/ml
■———■ NHS corresponding 1 CH$_{50}$/ml
△———△ NHS corresponding 2 CH$_{50}$/ml
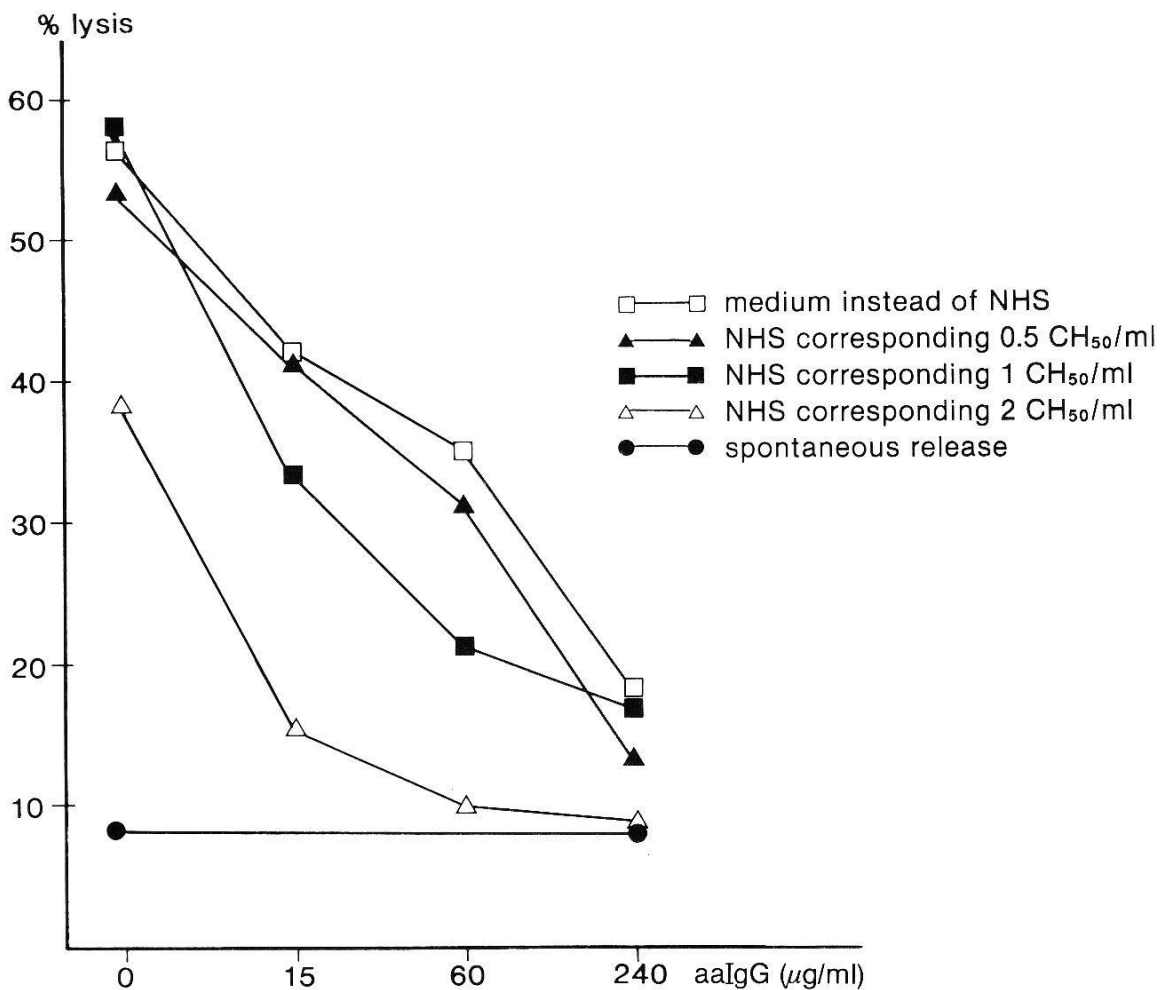●———● spontaneous release

aaIgG (μg/ml)

Fig. 5. The simultaneous effect of different concentrations of affinity purified antiamoebic antibodies (aaIgG) and fresh normal human serum as a source of complement on amoebae's cytotoxic activity after an incubation time of 30 min.

applying our experimental set up (a short term ^{51}Cr-release assay; Hudler et al., 1983) we are detecting features of ACA distinct from that described by Ravdin et al. (1980).

The addition of EGTA + Mg^{2+} left the inhibitory capacity of native human serum intact. This indicates that activation of the alternative pathway of complement in any case plays an important role in the inhibition of ACA.

All these serum components capable of inhibiting ACA either kill amoebae or are obviously interfering with recognition of the target cell and/or setting of the lethal hit (Figs. 3a and b). On the other hand, if the lethal message has been already delivered the events leading to target cell death cannot be influenced by serum components (Fig. 3b).

In a previous paper we reported that antibodies against amoebae inhibited the ACA in the sense that the isotope release was strongly reduced for at least 30 min (Hudler et al., 1984). This inhibitory capacity was attributed to redistribution events on the trophozoites surface and subsequent ingestion and shedding of the immune complexes. Although it seems conceivable that antibodies neither possess a direct cytotoxic/cytopathic effect on amoebae nor lead to lysis of trophozoites via activating the classical pathway of complement (Hudler et al., 1984), those antiamoebic antibodies added together with serially diluted serum exhibited an inhibition of ACA exceeding the values of the respective antibody concentration. This effect might be explained by the fact that opsonization of trophozoites with C3b bound via its metastable binding site leads to a transient immobilization of the surface membrane followed by redistribution phenomena and ingestion and shedding of C3b containing material (Scheiner, Stemberger, Wiedermann, unpublished results). These events closely resemble those observed with antibody-coated amoebae (Aust-Kettis and Sundquist, 1978; Biagi et al., 1966). It might well be that coating of the trophozoites with both IgG and C3b leads to amplification of the redistribution events which in turn are thought to be responsible for the inhibition of ACA. These observations strengthen the view that as well in the case of native serum as with antiamoebic antibodies the inhibition of ACA has to be distinguished from the cytotoxic events against trophozoites, e.g., via activation of the complement cascade. At present, we have prepared monoclonal antibodies against various strains of *Entamoeba histolytica* intending to isolate and characterize the molecules involved in the expression of ACA and to look for the interaction of these molecules with human antibodies and serum components.

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