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The occurrence and properties of E rosette inhibitory substance in the sera of malnourished children

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SUMMARY

In vitro sheep erythrocyte (E) rosette inhibitory activity was observed in the sera of nine out of 22 (41%) children with kwashiorkor, three of 15 (20%) marasmic children, neither of the two children with marasmic-kwashiorkor and in one of 42 (2%) well nourished control children. Sera of children with kwashiorkor containing the E rosette inhibitory substance did not inhibit *in vitro* rosette formations by autologous lymphocytes whereas rosette formations by homologous lymphocytes were inhibited. Inhibition of E rosette formation occurred when lymphocytes were pretreated with serum having the inhibitory substance before incubation with sheep red cells, but there was no such inhibition when sheep red cells were pretreated with the same serum before incubation with lymphocytes. The inhibitory substance was observed to be stable at 4°C up to about 1 week and migrated electrophoretically with the alpha-2 globulins. It was digested by papain. It is probable that the E rosette inhibitory substance demonstrated in the present study is attached to markers on T lymphocyte surfaces in some malnourished children thereby making the lymphocytes unreactive *in vitro* and presumably *in vivo* as well.

INTRODUCTION

Impaired transformation of lymphocytes of malnourished children in phytohaemagglutinin (PHA) cultures in the presence of autologous plasma have been reported by some workers (Geefhysen *et al.*, 1971; Grace, Armstrong & Smythe, 1972). When challenged with mitogens or antigens, the *in vitro* proliferation of lymphocytes of healthy donors was also shown to be inhibited by sera or plasma from malnourished subjects (Chandra, 1974; Heyworth, Moore & Brown, 1975; Moore, Heyworth & Brown, 1977; Beatty & Dowdle 1979). In the present studies the effect on E rosette formation (which is a method of determining the subpopulation of T lymphocytes in blood) of sera from malnourished and well nourished control children are investigated.

MATERIALS AND METHODS

The investigations were performed on 39 malnourished Nigerian children, comprising 22 children with kwashiorkor, 15 with marasmus and two with marasmic-kwashiorkor. The malnourished children came mainly from Ibadan and environs to the General Out Patients Clinic from where they were referred to the Paediatric Gastroenterology/Nutrition Clinic both at the University College Hospital (UCH), Ibadan, Nigeria. A large number of children with primary protein-energy malnutrition were seen at the Gastroenterology/Nutrition Clinic where this study was carried out. The children who had associated gastrointestinal and other infections were identified by careful

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clinical examination and laboratory investigations. Stools were examined and cultured in those with diarrhoea. The urine samples were examined and the blood cultured in those with hypo- or hyperthermia. Chest X-rays were performed in all cases to identify those with occult chest infections. All the children with detected infections were excluded from the present study.

Included in the study were 42 age matched well fed control children from the same community as the malnourished children and attending the same 'Paediatric Clinic' for minor ailments or admitted to the ward for minor surgical procedures such as release of burns contractures, herniorrhappy and correction of urogenital anomalies such as hypospadias.

Five millilitres of blood was collected from each patient by venipuncture. Serum was separated asceptically soon after the clot had retracted. It was either used immediately or stored at $-20°C$ until needed. In case of the control children, the blood was collected as part of other essential investigations for their management.

Lymphocyte preparation. Lymphocytes were prepared by the Ficoll-Hypaque density gradient method of Boyum (1978).

E rosette inhibition test. Two 10×60 nm plastic tubes were set up per test sample. Into the tubes were placed 0·25 ml of 4×10^6 lymphocytes per ml and 0·25 ml of thrice washed 0·5 per cent sheep red cells. Into one of the tubes was added 0·1 ml of neat inactivated, absorbed malnourished or control child's serum. To the other was added a 1/20 dilution (in fetal calf serum) of the same serum. In addition, a reference control tube contained 0·25 ml of 4×10^6 lymphocytes per ml, 0·25 ml of 0·5 per cent sheep red cells and 0·1 ml of inactivated adsorbed fetal calf serum. The tubes were incubated at 37°C for 5 min. They were centrifuged at 200 ***g*** for 4–5 min and then incubated at 4°C overnight. The next day, the pellet was gently resuspended. Two hundred lymphocytes were counted in each sample using Fuchs–Rosenthal counting chamber. The percentage of E rosette forming lymphocytes in the presence or absence of the test serum was determined. A rosette forming lymphocyte was defined as one that bound three or more sheep red cells.

E rosette inhibition test after incubation of sera from kwashiorkor patients with autologous as well as homologous lymphocytes. E rosette inhibition assay was performed as described above. Kwashiorkor serum known to contain E rosette inhibitory substance was incubated (neat or diluted) with autologous as well as homologous lymphocytes.

Effect of pre-treating normal lymphocytes with serum containing E rosette inhibitory substance before incubating with sheep red cells. In each of three 10×60 mm plastic tubes was added 0·1 ml of neat or diluted (in fetal calf serum) heat inactivated adsorbed serum from kwashiorkor patients containing E rosette inhibitory substance. To each tube was added 0·25 ml of 4×10^6 control child's lymphocytes per ml. The mixture was incubated at 37°C for 5 min and later at 4°C for 55 min. The mixture was centrifuged at 200 ***g*** for 4–5 min. The cell pellet was washed twice with Krebs–Ringer phosphate buffer pH 7·4. The supernatant was discarded. The pellet was resuspended in 0·25 ml of 0·5 per cent sheep red blood cells and 0·1 ml of inactivated adsorbed fetal calf serum. A reference control tube was also set up in which fetal calf serum replaced the test serum. The test and control tubes were incubated at 37°C for 5 min and centrifuged at 200 ***g*** for 4 min. The percentage E rosettes were read after overnight incubation at 4°C.

Effect of pre-treating sheep red blood cels with serum containing E rosette inhibitory substance before incubation with lymphocytes. A procedure similar to that described above for pre-treated lymphocytes was employed. In this case however, the sheep red blood cells were first incubated with the E rosette inhibitory substance (instead of the lymphocytes) before incubation with the lymphocytes.

Effect of temperature on the inhibitory activity of pooled serum. Sera from five children having kwashiorkor and two marasmic children which were known to have inhibitory activity were heat inactivated, adsorbed with sheep erythrocytes and pooled. The pooled serum was diluted 1 in 40 with phosphate buffered saline (PBS) and distributed in 0·5 ml volumes in 21 sterile covered plastic tubes labelled with the appropriate temperatures and proposed periods of incubation. The temperatures chosen were $-20°C$, 4°C, 37°C. 46°C 56°C, 65°C and 100°C. The periods of incubation were 30 min 2 hr, and 24 hr. Each sample was removed from incubation at appropriate intervals and tested for the presence of E rosette inhibitory activity. Heat inactivated fetal calf serum replaced the serum in the reference control.

Effect of period of storage at 4°C on the inhibitory activity of serum. Sera from five of the children with kwashiorkor were pooled. The pooled serum was distributed in 0·5 ml aliquots into seven labelled tightly covered plastic tubes. The tubes were immediately stored at −20°C after heat inactivation at 56°C for 30 min and absorption with sheep red cells.

At 4 weeks, 3 weeks, 2 weeks, 1 week, 5 days and 1 day prior to the day of testing, the appropriate aliquots were removed and stored at 4°C. On the day of test (day zero) the last sample was removed from storage (−20°C). All the samples were tested for the presence of inhibitory activity.

Membrane dialysis (against PBS). Equal volumes of sera containing inhibitory substance were pooled from two kwashiorkor children. The pooled serum (6 ml volume) was dialysed overnight in 0·01 M.PBS, pH 7·2.

Sephadex G-200 column chromatography. Three millilitres of the pooled serum was fractionated by passage through a 90 × 2·5 cm glass column (Pharmacia, Sweden) containing Sephadex G-200 in PBS, pH 7·2. The procedure was repeated using 3 ml of pooled serum from two control children.

The eluates were each concentrated about 10 times by sucrose dialysis and tested for the presence of E rosette inhibitory activity. All eluates containing E rosette inhibitory activity were pooled and further concentrated to 2 ml. The corresponding eluates from the control serum fractionation were also pooled and concentrated to the same volume (2 ml).

Papain digestion and paper electrophoresis. To 0·5 ml of pooled concentrated Sephadex G-200 eluates from kwashiorkor sera containing the inhibitory substance was added 0·5 ml of papain (0·5% w/v) in 0·1 M sodium phosphate buffer pH 7·0 containing 0·1 M cystein and 0·002 M ethylene diamine-tetra acetic acid. 0·5 millitres of pooled concentrated eluates from the control sera was similarly treated. The papain treated and untreated pooled eluates were fractionated by cellulose acetate zonal electrophoresis.

Glycoprotein and lipoprotein staining of agarose zonal electrophoretic strips. The kwashiorkor serum having inhibitory activity and corresponding eluates from control serum were fractionated electrophoretically on 1 per cent agarose in 0·05 M barbitone buffer pH 8·6. The glycoprotein was stained using Periodic acid-Schiff method whilst the lipoprotein was stained by Sudan Black B.

RESULTS

The coefficient of variation of repeated measurements of E rosette formation was computed using the formula 100 × (s.d.)/mean. This was found to range from ±4 to ±9% with a mean of ±6 %.

Percentage of E rosette formation of any cell mixture containing test serum that was lower than that of the reference control (fetal calf serum) by over 18 E rosettes (3 × mean coefficient of variation) was regarded as showing some inhibition.

A comparison of the proportions of malnourished with control children having serum inhibitory substance shows that nine out of 22 (41%) of the children with kwashiorkor, three out of 15 (20%) of those with marasmus and only one out of 42 (2%) of the well fed (control) children

Table 1. Effect of pre-treating lymphocytes of a control child 'J' with serum of a kwashiorkor child 'A' (having inhibitory substance) before incubation with sheep red cells

Serum dilution	Percentage E rosettes
Neat	27%
1/40	23%
1/1280	43%
Reference control (fetal calf serum)	49%

Table 2. Effect of pre-treating sheep red blood cells with serum of a kwashiorkor child 'A' containing inhibitory substance before incubation with the lymphocytes of control child 'J'

Serum dilution	Percentage E rosette
Neat	45%
1/40	52%
1/1280	50%
Reference control (fetal calf serum)	49%

Table 3. Effect of storage period at 4°C on the inhibitory activity of pooled serum from five children with kwashiorkor who were known to have serum E rosette inhibitory substance

	Percentage E rosette* values (average of duplicate tests).						
Serum dilution	Day zero	1 day	5 day	1 week	2 weeks	3 weeks	4 weeks
Neat	16%	18%	20%	19%	33%	36%	36%
1/40	13%	12%	15%	17%	36%	37%	38%
1/1280	19%	31%	32%	32%	32%	34%	36%
Reference control (fetal calf serum) 40%							

* The lymphocytes were obtained from a healthy well fed control child.

Table 4. Effect of temperature on the inhibitory activity of pooled serum of seven patients who were previously demonstrated to have inhibitory substance

	Percentage E rosette* values (average of duplicate tests). Temperature						
(a) After 30 min incubation							
Serum dilution	−20°C	4°C	37°C	46°C	56°C	65°C	100°C
1/40	20%	21%	24%	23%	29%	59%	63%
Reference control (fetal calf serum) 57%							
(b) After 2 hr incubation							
1/40	19%	22%	20%	32%	40%	59%	59%
Reference control (fetal calf serum) 57%							
(c) After 24 hr incubation							
1/40	30%	31%	32%	60%	58%	59%	57%
Reference control (fetal calf serum) 57%							

* Lymphocytes were obtained from one control subject for results in Tables 4a, b & c.

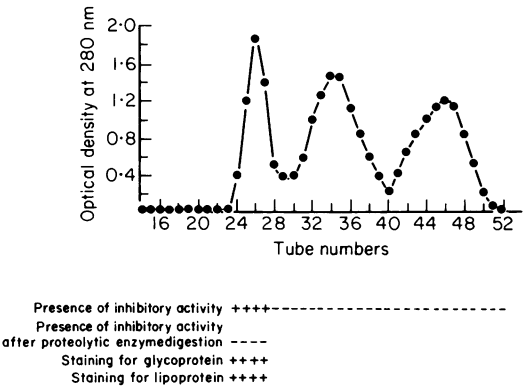

Fig. 1. Sephadex G-200 elution pattern of pooled kwashiorkor sera containing E-rosette inhibitory substance(s).

showed the presence of E rosette inhibitory substance. Neither of the two children with marasmic-kwashiorkor included in the study had any serum E rosette inhibitory substance. The control children were significantly different from the kwashiorkor children ($\chi^2 = 16{,}256$, $P < 0{\cdot}005$) as well as the marasmic children ($\chi^2 = 5{\cdot}2583$, $P < 0{\cdot}025$) in the proportions of children showing presence of inhibitory substance.

Sera from kwashiorkor children having E rosette inhibitory substance were observed not to inhibit their own (autologous) T lymphocytes rosette formation *in vitro*. The sera, however, inhibited the E rosetting of control children's (homologous) lymphocytes. Pre-treatment of the T lymphocytes (with serum containing the inhibitory substance) before incubation with sheep red cells caused inhibition of the E rosette formations (Table 1). In contrast, when sheep red cells were pre-treated with serum containing the inhibitory substance, before incubation with lymphocytes, there was no inhibition of the E rosette formation (Table 2). The serum E rosette inhibitory activity of positive sera was maintained at 4°C up to 5–7 days. Thereafter the inhibitory substance in the sera became unstable (Table 3).

The E rosette inhibitory activity of the sera was retained up to a temperature of 56°C when incubated for 30 min or 2 hr. The inhibitory activity was lost when the sera were incubated for 30 min or more at a temperature of 65°C or above (Table 4). After 24 hr incubation at 37°C and below,

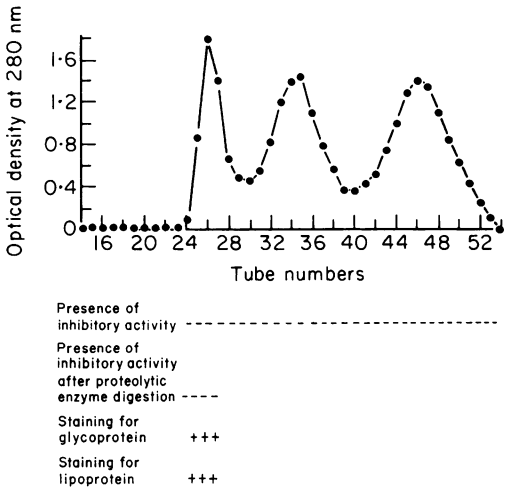

Fig. 2. Sephadex G-200 elution pattern of pooled control sera having no E-rosette inhibitory substance(s)

the inhibitory activity of the serum was still demonstrable. However, inhibitory activity was lost after 24 hr incubation at temperature of 46°C and above (Table 4).

Dialysis of the serum containing the inhibitory activity in PBS had no adverse effect on the inhibition. At a forty-fold dilution, the dialysed serum was observed to be more strongly inhibitory than undialysed serum.

Fig. 1 shows the optical density readings at 280 nm of Sephadex G-200 eluates of the pooled serum of kwashiorkor children containing inhibitory activity against corresponding tube numbers. The ten-fold concentrated pooled eluates showed the presence of E rosette inhibitory activity in tubes 24–27.

The optical density readings of Sephadex G-200 eluates of pooled control serum is shown in Fig. 2. None of the concentrated eluates of the pooled control serum showed inhibitory activity.

The E rosette inhibitory activity of the eluates was observed to migrate with the alpha-2 globulins on cellulose acetate electrophoresis. Papain was observed to digest the inhibitory substance as well as destroy the inhibitory activity of the eluates on E rosette formation.

The concentrated Sephadex G-200 eluates of kwashiorkor sera containing E rosette inhibitory substance showed the presence of glycoprotein and lipoprotein. Similarly the corresponding eluates from the control serum without E rosette inhibitory activity also showed the presence of glycoprotein and lipoprotein. The stainings of the glycoprotein and lipoprotein were however less intense in the eluates of control sera than in those of kwashiorkor sera having E rosette inhibitory substance.

DISCUSSION

In the present study, it was observed that sera from some malnourished children inhibited sheep erythrocyte (E) rosette formation by lymphocytes. The reason why the sera from the two children with marasmic-kwashiorkor did not show any inhibition is not known. It is probably because the number studied was small. In addition, it is also not known why E rosette inhibition was detected only in 12 out of 39 sera. It is possible that the inhibitory substance was not detected in the remaining 27 malnourished children either because of low sensitivity of the method of assay, or because the substance has been largely adsorbed *in vivo* by autologous lymphocytes. Children with overt infections were excluded from the study and therefore the detection of inhibitory substance in the 12 malnourished children could not be due to clinically overt infections at the time of blood sampling. The possibility of convalescence from recent infections can however not be ruled out in all the children studied.

It was demonstrated in the present study that the sera of malnourished children having inhibitory substance did not inhibit their own (autologous) lymphocytes *in vitro* whereas the E rosette formation of other children's (homologous) lymphocytes were inhibited. This would indicate that such malnourished children's T lymphocytes had been optimally inhibited *in vivo*. This could be as a result of either *in vivo* binding of T lymphocyte receptors with the inhibitory substance or the effect of stearic hindrance of T lymphocyte receptors by the inhibitory substance. Such a T lymphocyte with attached inhibitory substance would be denser than an active 'normal' T lymphocyte having no attached substance.

Very recently, Mahalabanis *et al.* (1979) showed that not only was there a reduction in the percentage of E rosette forming T lymphocytes in children with kwashiorkor but there was also an increase in their density. These workers also observed that the non-E rosetting lymphocytes ('null cells') of kwashiorkor children also settled 'in the fraction with the highest density'. The B lymphocytes of the kwashiorkor children were however observed to be similar in density to those of control children. Such observations coupled with the findings in the present study would support an hypothesis that the T lymphocytes of some kwashiorkor children have an inhibitory substance(s) attached to markers on their cell surfaces *in vivo* thereby making them denser than normal T lymphocytes not having any such attachments. These attachments would affect the binding of T cell markers to receptors on sheep red cells *in vitro*. Massive attachments of the inhibitory substance to

most or all the available T cell surface markers would render these T lymphocytes unreactive with sheep red cell receptors. T lymphocytes with large attachments of the inhibitory substance would appear as 'null cells' with high density. Since the percentage of null cells was always obtained by the difference between the total lymphocytes and the sum of the T and B lymphocytes, such *in vivo* inhibited T lymphocytes would be recorded as null cells. This could account for the high percentage of null cells that have been reported in kwashiorkor (Schopfer & Douglas, 1976; Chandra, 1977) and which we have also observed in our study (Salimonu, 1980). It is not known whether such attachments would be limited to only the T lymphocytes.

The *in vitro* inhibition of E rosette formation observed in a considerable proportion of malnourished children in the present study may have its implications *in vivo*. In the E rosette test, low E rosette formation of peripheral T lymphocytes is considered to be an indication of T lymphocyte depletion, a situation that has been reported in kwashiorkor by several authors (Neumann *et al.*, 1977) and which has also been confirmed by us (Salimonu, 1980). Furthermore, it is known that there is impaired lymphocyte transformation following mitogenic (Chandra, 1972; Jose *et al.*, 1975) and antigenic (Jose, Welch & Doherty, 1970) challenges. Also the *in vitro* stimulations of lymphocytes of healthy donors by mitogens and antigens are inhibited by sera from malnourished children (Chandra, 1974; Heyworth, Moore & Brown, 1975). It is possible that the same inhibitory substance identified in the present study is also responsible for the inhibition of T lymphocyte proliferation following mitogenic and antigenic stimulations thereby resulting in the impaired cellular immune responses that have been documented in malnourished children by several workers (Geefhuysen *et al.*, 1971, Grace *et al.*, 1972, Moore *et al.*, 1977, Beatty & Dowdle, 1979).

There is, however, evidence that thymosin can increase E rosetting and induce null cells to form rosettes (Jackson & Zaman, 1980). In addition low serum thymic hormone activities have been reported in protein depletion in both man (Chandra, 1979), and experimental animals (Heresi & Chandra, 1980). These findings would suggest that there may be many factors responsible for the observed diminution in the percentage of the peripheral E rosetting lymphocytes in protein calorie malnutrition.

There are a number of possibilities as to the exact nature of the 'substance' responsible for the serum E rosette inhibitory activity. It is possible that the inhibitory substance may be one or more of the acute phase proteins. These proteins include C-reactive protein, alpha-1 antitrypsin, alpha-2 macroglobulin, fibrinogen, 3rd component of complement, alpha-1 acid glycoprotein, and haptoglobin. These proteins are usually elevated in most inflammatory conditions. Most of them also migrate between alpha-1 and beta globulin regions as was observed for the inhibitory substance. Razban *et al.* (1975) investigating the acute phase proteins, showed that C-reactive protein was present in significantly higher quantities in 93% of the malnourished children studied. Only 16% of the control children had C-reactive proteins in their sera and the quantities in such sera were small (Razban *et al.*, 1975). In addition, it is known that C-reactive protein inhibits lymphocyte transformation *in vitro* (Hokama *et al.*, 1973) and, it is also probable that it inhibits E rosette formation.

Infants who suffered from intra-uterine growth retardation and those with post-natal nutritional depletion have been observed to have elevated serum alpha-feto-protein (Chandra & Bhujwala, 1977). This protein has been shown to suppress immune responses both *in vitro* (Figueredo, Palomino & Ortiz, 1979), and *in vivo* (Chandra & Bhujwala 1977). It is therefore not impossible that alpha-feto-protein could be the serum inhibitory substance observed in the malnourished children. Alternatively, its presence could enhance the inhibitory activity of the serum. The inhibitory activity could also be due to the deficiency of a promoting factor as postulated by Beatty & Dowdle (1979).

Furthermore, normal human plasma is known to contain an alpha-2 glycoprotein which inhibits the transformation of lymphocytes by phytohaemagglutinin (Cooperband *et al.*, 1968). This protein may be increased in some disease states (Riggio *et al.*, 1969). In addition, it was observed in the present study that the E rosette inhibitory substance migrated as an alpha-2 protein staining positively for glycoprotein. Previous studies have however demonstrated decreased alpha-2 glycoprotein in severe states of kwashiorkor (Coward, Whitehead & Coward, 1972).

Alpha-2 glycoprotein is therefore not likely to be responsible for the E rosette inhibition observed in the present study.

Infections with gram negative bacteria are common in severe protein calorie malnutrition (Phillips & Warton, 1968; Graham, 1972). Endotoxins constitute a portion of the cell wall of gram negative bacteria and may be found in various body fluids during infection with these organisms. Oberle, Graham & Levin, (1974) showed that a large percentage of children with protein calorie malnutrition have evidence of circulating endotoxins and this may be responsible for the E rosette inhibitory activity of the serum in these children.

During the course of infections, circulating antibodies are formed. These antibodies normally react with the specific antigens to form soluble immune complexes. There is evidence that circulating immune complexes may be involved in the pathogenesis of some diseases such as malaria (Lambert & Houba, 1974), measles (Charlesworth *et al.*, 1976), hepatitis (Madalinski & Bragiel, 1979) and thyroiditis (Brohee *et al.*, 1979). It is pertinent to add also that in several of these infections or diseases in which immune complexes are involved, low E rosette formations have been reported. These include malaria (Wells *et al.*, 1979), systemic lupus erythematosus (Hurd & Giuliano, 1975) and rheumatoid arthritis (Yu & Peter, 1974).

Similarly, children with protein calorie malnutrition are highly prone to infections and would therefore have circulating antibodies in their sera. These circulating antibodies in the presence of excess antigen(s) may facilitate the development of soluble immune complexes in such children. In addition, in common with other patients known to have circulating immune complexes, children with protein calorie malnutrition have low E rosette forming lymphocytes (Chandra, 1977; Newmann *et al.*, 1977; Schopfer & Douglas, 1976; Salimonu, 1980). It is possible that the low proportions of circulating T lymphocytes reported in the malnourished children, as well as in these other disease conditions, are the results of inhibitory effects of immune complexes on E rosette formation. This postulate would corroborate the findings of De Cock, Decree & Verhaegen (1978), who observed that aggregated IgG (which behaves as an immune complex) inhibited E rosette formation by lymphocytes of healthy subjects and cancer patients. The findings in the present study that the inhibitory substance is a high molecular weight protein fits in with the properties of immune complexes. That the substance does not keep indefinitely at 4°C might be as a result of the dissociation of the complexes.

In summary, it seems probable that the E rosette inhibitory substance (s) present in the blood of some malnourished children either sterically hinders or cross reacts with the T lymphocyte receptor *in vivo* and *in vitro*, thus reducing the proportion of T lymphocytes that can form E rosette with sheep erythrocytes *in vitro*. From available evidence, it appears that the E rosette inhibitory substance is likely to be either soluble immune complexes, endotoxin or alpha-2 macroglobulin.

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