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Surface Receptors on Neutrophils and Monocytes from Immunodeficient and Normal Horses

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Summary. Surface receptors on peripheral blood neutrophils and monocytes from normal and immunodeficient horses have been studied. Sheep erythrocytes (SRBC) coated with IgG, IgM, and complement, but not IgG(T), readily bound to normal equine monocytes and neutrophils. More than 4000 molecules of IgG were required to sensitize each SRBC for adherence to monocytes, and more than 12,000 molecules were required for adherence to neutrophils. Young horses with a severe combined immunodeficiency had an almost total absence of lymphocytes, but normal numbers of monocytes and neutrophils. The number of receptors for immunoglobulin, complement, and phytolectin on monocytes and neutrophils from immunodeficient animals were similar to those on the cells of normal horses. Although the precursor cells of lymphocytes of horses with combined immunodeficiency appear to be defective, no defect in the other cellular products of the bone marrow were apparent.

INTRODUCTION

Neutrophil and monocyte phagocytosis and participation in the immune response are related to the presence of surface receptors for complement and various classes of immunoglobulin (Rabinovitch, 1970). Apparent defects in surface receptors have been associated with abnormalities of host defence, involving: (1) disorders of chemotaxis associated with cellular immune deficiency (Synderman, Altman, Frankel and Blaese, 1973; Clark, Root, Kimball and Kirkpatrick, 1973) and sex-linked congenital agammaglobulinaemia (Steerman, Synderman, Leikin and Colten, 1971); (2) absence of monocyte receptors in the Wiskott–Aldrich syndrome (Spitler, Levin, Fudenberg and Huber, 1972); and (3) the inability of monocytes of anergic patients to respond to migration inhibition factor (Louie and Goldberg, 1972).

A defect in the immune system of young horses has recently been described (McGuire and Poppie, 1973; McGuire, Poppie and Banks, 1974). The abnormality is most like severe combined immunodeficiency of children with marked lymphopenia, absence of immunoglobulin production, and absence of lymphocytes in all lymphoid organs, including the thymus. Affected horses die from a variety of viral, bacterial, and fungal diseases. The studies reported here were undertaken to define the basic characteristics

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of immunoglobulin, complement and phytolectin receptors of immunodeficient and normal equine monocytes and neutrophils.

MATERIALS AND METHODS

Antisera

Four horses were immunized with washed sheep red blood cells (SRBC). Each horse was initially injected subcutaneously with 2 ml of a 30 per cent suspension of SRBC suspended in 2 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan). The animals were also given 2 ml of a 30 per cent suspension intravenously. Three additional weekly subcutaneous injections of 2 ml of a 30 per cent suspension were given. Sera were collected 2 days following the second immunization and 7 days following the last injection, and designated Ab1 and Ab2, respectively. Sera were pooled in equal amounts from the four horses, inactivated at 56° for 30 minutes, and stored at −20°C.

Eluates of anti-SRBC antibody were prepared by mixing 1 ml of washed, packed SRBC with 5 ml of Ab1 or Ab2 antisera, and then incubated at 37° for 1 hour and at room temperature for 2 hours. The SRBC were deposited by centrifugation, washed three times, and resuspended in 2 ml of phosphate-buffered saline (PBS). The suspensions were heated at 56° for 20 minutes, SRBC quickly centrifuged, and the supernatant fluids collected. To determine the immunoglobulin classes involved in the various reactions, whole serum or antibody eluted from SRBC was separated into IgM, IgG and IgG(T) immunoglobulin classes by techniques that have been previously described (McGuire and Crawford, 1972). The purity of the preparations was ascertained by agglutination of sensitized SRBC with specific anti-heavy chain antisera to equine IgM, IgG, IgG(T), IgA, and aggregating immunoglobulin. Monovalent Fab fragments of IgG were prepared by papain treatment, DEAE fractionation and, finally, separation on Bio gel A 0·5 (Bio-Rad Laboratories, Richmond, California) to remove undigested molecules (Porter, 1959).

Sensitization of SRBC

SRBC were collected weekly, washed three times with Hanks's balanced salt solution (HBSS) and stored at 4° in HBSS. One-millilitre portions of 1 per cent SRBC were mixed with 1 ml of various dilutions of antisera or purified fractions and incubated at 37° for 30 minutes. The cells were washed twice and resuspended in 1 ml of HBSS. SRBC sensitized in this manner were designated EAb1, EAb2, EIgM, EIgG, and EIgG(T). In those studies in which complement (C) was added, 1-ml aliquots of SRBC, with the antibody attached, were incubated with 0·5 ml of 1:50 dilution of fresh horse serum. This mixture was incubated at 37° for 30 minutes, washed twice with HBSS and then resuspended in 1 ml. Glutaraldehyde-treated erythrocytes were prepared by mixing 1 ml of packed SRBC with 10 ml of 1·5 per cent glutaraldehyde for 24 hours at 4°. SRBC were then washed twice and a 1 per cent suspension made in HBSS.

The number of molecules of IgG on the SRBC was determined by quantitative radioimmunoassay (Rabellino, Colon, Grey and Unanue, 1971). Purified equine IgG was used to obtain a standard inhibition curve (McGuire and Crawford, 1972). The protein content of the IgG standard was determined by the method of Lowry, Rosebrough, Farr and Randall (1951). Sensitized erythrocytes were washed four to eight times. One

portion of the washed cells was examined by radioimmunoassay and another portion examined for monocyte and neutrophil binding.

Leucocyte preparations

Peripheral blood leucocytes were studied from three groups of horses; normal adults; parents of immunodeficient horses; and horses with combined immunodeficiency (CID). The latter animals were identified, as previously described, on the evidence of lymphopenia, absence of serum IgM, and absence of lymphoid tissue (McGuire and Poppie, 1973; McGuire *et al.*, 1974). Peripheral blood was allowed to sediment for 30 minutes, and the leucocyte-rich plasma harvested. Further separation of cells was made by layering leucocytes onto Hypaque–Ficoll separation fluid and centrifuging at 400 ***g*** for 40 minutes, according to the method of Boyum (1968). The pellet formed by this procedure contained neutrophils and erythrocytes, while the lymphocytes and monocytes remained at the plasma-separation fluid interphase. Following washing, the cells used for monocyte assay were resuspended in HBSS with 20 per cent normal horse serum. Preparations used for neutrophil study were resuspended in HBSS alone.

Assay of leucocyte receptors

Monocytes and neutrophils were studied while attached to glass. Open chambers were formed with Lucite rings attached to glass coverslips, according to the method of Berken and Benacerraf (1966). Cell preparations containing $2–10 \times 10^5$ monocytes or neutrophils were placed in these chambers, incubated at 37° for 30 minutes, and then washed three times to remove nonadherent cells. Phytolectin binding to leucocytes was determined by adding 100 μg of phytohaemagglutinin (PHA-P, Difco Laboratories, Detroit, Michigan) or 200 μg of concanavalin A (Con A) (Sigma, St Louis, Missouri) in 0·1-ml volumes to the leucocyte chambers. SRBC preparations were added to the leucocytes and incubated at 37° for 1 hour. The coverslips were washed three times and then examined as wet mounts in the presence of a 0·002 per cent Neutral Red solution, or the coverslips were stained with Giemsa reagent after removal of the Lucite rings. The degree of SRBC binding to monocytes and neutrophils was scored according to the following scale: + + + +, rosettes on 70–90 per cent of cells; + + +, SRBC on almost every cell and rosettes on about 50 per cent of cells; + +, SRBC on 50 per cent of cells; +, SRBC on some cells in every high power field. In some cases, the percentage of leucocytes with three or more SRBC attached, or ingested, was determined by counting 200–300 leucocytes.

RESULTS

NORMAL EQUINE MONOCYTES AND NEUTROPHILS

The two leucocyte preparations were characterized as follows. Those designated monocytes were cells which were adherent to glass, accumulated Neutral Red in areas adjacent to the nucleus, were phagocytic, and contained a bilobed nucleus and grey basophilic cytoplasm stippled by tiny granules when stained. These cells also differed in density from neutrophils in the Hypaque–Ficoll separation fluid used. Neutrophils were adherent to glass, phagocytic and had the distinct morphological characteristics of this cell type upon staining.

Experiments showed that monocytes and neutrophils bind to SRBC coated with late

TABLE 1

SHEEP RED BLOOD CELLS (E) SENSITIZED WITH ANTIBODY AND COMPLEMENT (C) BINDING TO EQUINE MONOCYTES AND NEUTROPHILS

Erythrocyte preparation	Binding to leucocytes	
	Monocytes	Neutrophils
E	0	0
EAb1*	0 to +	0
EAb2†	++++	++++
EAb1+C	++++	++++
EAb2+C	++++	++++
E+C	0	0

* Ab1 = anti-SRBC antibody collected early in immunization and containing predominantly IgM antibodies.
† Ab2 = anti-SRBC antibody collected late in immunization and containing both IgG and IgG(T) antibodies.

antibody (Ab2) or complement (Table 1). SRBC incubated with Ab2 (EAb2) readily attached to all monocytes and neutrophils, often with subsequent phagocytosis. Binding of EAb2 to monocytes and neutrophils could be inhibited by free serum (7 mg), gamma-globulin fractions of horse serum (3 mg), or 0·01 M EDTA. SRBC coated with early antibody (EAb1) did not bind to neutrophils and only occasionally to 1–10 per cent of the monocytes. When fresh horse serum, as a complement source, was incubated with EAb1, the red cells formed large rosettes with all monocytes and neutrophils. Red cells coated with Ab1 did not bind if the complement source was heated at 56° for 30 minutes, or if EDTA (0·01 M) was mixed with complement during incubation with the Ab1-coated erythrocytes. EAb1 with complement did not bind if EDTA (0·1 M) was present, but did bind in the presence of serum or gamma-globulin.

Immunoglobulin M was isolated from Ab1 while IgG and IgG(T) were isolated from Ab2 and the eluates of SRBC treated with Ab2. These fractions were shown to contain only their respective class by reaction with monospecific antisera in gel diffusion and indirect Coombs' reactions. Results of binding of these immunoglobulin classes to leucocytes is summarized in Table 2. Red cells coated with IgG attached to neutrophils and monocytes and were phagocytozed. SRBC coated with IgM bound to a small percentage of monocytes, while IgG(T) did not bind to the leucocytes. When complement

TABLE 2

BINDING TO HORSE MONOCYTES AND NEUTROPHILS OF SHEEP RED BLOOD CELLS (E) SENSITIZED WITH SPECIFIC IMMUNOGLOBULIN CLASSES OF ANTIBODY AND COMPLEMENT (C)

E with sensitizing immunoglobulin class	Binding to leucocytes	
	Monocytes	Neutrophils
EIgG	++++	++++
EIgM	0 to +	0
EIgG(T)	0	0
EIgG+C	++++	++++
EIgM+C	++++	++++
EIgG(T)+C	0	0

was added to red cells sensitized with IgM, IgG and IgG(T), rosettes were formed on monocytes and neutrophils with IgM and IgG, but not the IgG(T) immunoglobulin class.

Anti-SRBC of the IgG class was treated with papain, monovalent Fab fragments isolated, and reacted with SRBC. SRBC sensitized by Fab did not bind to monocytes or neutrophils, although the Fab fragments were shown to be present by indirect Coombs' reactions.

The number of IgG antibody molecules on the SRBC necessary for attachment to monocytes and neutrophils is shown in Fig. 1. The minimum number of molecules detect-

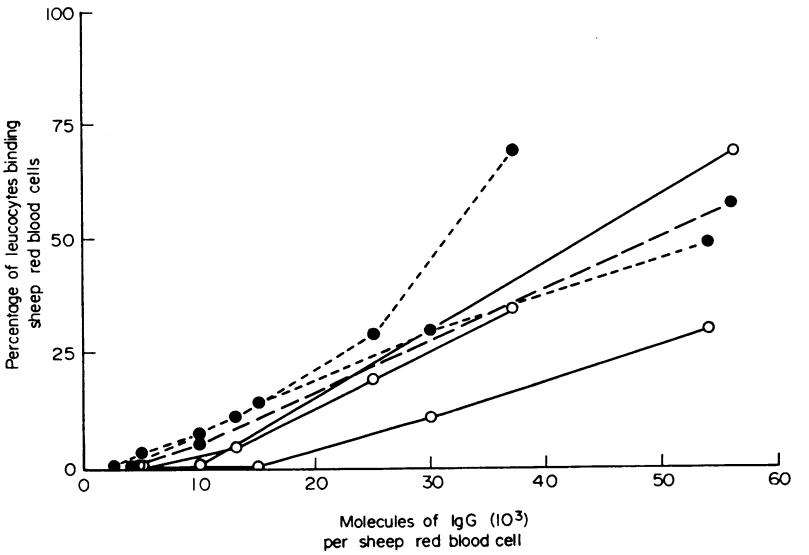

FIG. 1. The percentage of monocytes (● – – ●) or neutrophils (○——○) binding sensitized sheep red blood cells (SRBC) is plotted against the number of molecules of IgG antibody present on the SRBC. At lower amounts of antibody, a small percentage of monocytes continue to bind to the sensitized SRBC, while there are fewer SRBC binding to neutrophils.

able was 4000 for monocytes and 13,000 for neutrophils. A greater amount of attachment and subsequent phagocytosis was noted with $3–4 \times 10^5$ molecules per SRBC.

MONOCYTE AND NEUTROPHILS OF IMMUNODEFICIENT AND PARENT ANIMALS

The immunoglobulin, complement, and phytolectin receptors of two immunodeficient horses and four parents of affected horses were examined. Examination of peripheral leucocytes of affected animals showed only 0–3 per cent of the white blood cells were lymphocytes, while non-affected animals of this age have 20–50 per cent lymphocytes. Total leucocyteco unts ranged from 4 to 31×10^3 per mm^3. The monocytes of the affected animals were 1–3 per cent of the total leucocytes present, while neutrophils made up 94–98 per cent of the white blood cells. As with total leucocyte counts, the number of erythrocytes was within the range of non-affected young horses (McGuire *et al.*, 1974). The deficiency of lymphocytes was associated with an apparent failure of immunoglobulin production, as determined by an absence of serum IgM; and, before death, a deficiency

of IgG, IgA and IgG(T). The numbers of each leucocyte-type and the erythrocytes of parents of affected foals were within normal ranges. The percentage of affected and parent leucocytes binding sensitized SRBC was compared with the binding by normal horse leucocytes (Table 3). SRBC sensitized with late antiserum (Ab2) and early antiserum with complement (Ab1 + C) readily attached to the affected and parent neutrophils and monocytes, frequently leading to phagocytosis. No significant difference in the amount of binding of leucocytes from the groups of horses was detected by this technique. Other experiments showed a similar amount of binding of CID, parent and normal horse leucocytes to gluteraldehyde-treated SRBC, PHA and Con A.

TABLE 3

PERCENTAGE OF IMMUNODEFICIENT, PARENT, AND NORMAL HORSE MONOCYTES AND NEUTROPHILS BINDING TO SENSITIZED SHEEP RED BLOOD CELLS (E)

Erythrocyte preparation	Monocytes				Neutrophils			
	Immunodeficient*		Parents	Normal	Immunodeficient*		Parents	Normal
	No. 1	No. 2			No. 1	No. 2		
E	0	0	0	0	2	0	0	0
EAb1	28	0	0	0	0	0	0	0
EAb2	62	54	31 ± 4†	35 ± 12†	48	25	55 ± 25†	39 ± 18†
EAb1 + C	62	100	98 ± 8	92 ± 9	56	100	99 ± 1	98 ± 2
E + C	0	0	0	0	0	0	0	0

* The number of bound cells of two immunodeficient horses.
† The mean ± s.e. of four parents or nonaffected horses.

DISCUSSION

SRBC coated with late-forming equine anti-SRBC (Ab2), consistently bound to equine monocytes and neutrophils. This binding could be blocked by serum, gamma-globulins, and EDTA, indicating that free immunoglobulins compete for the receptor sites and that Ca^{2+} and/or Mg^{2+} ions are needed for attachment. It was observed that, of the purified classes of anti-SRBC antiserum, IgG most readily attached to monocytes and neutrophils, while IgG(T), a subclass of equine IgG (McGuire *et al.*, 1972) did not bind. Treatment of IgG antibody with papain, which removes the Fc portion of the immunoglobulin molecule and yields monovalent fragments (Porter, 1959), destroyed the ability of IgG anti-SRBC to bind to leucocytes. It has been reported that human IgG1, IgG3, and occasionally IgG2 (Abramson and Schur, 1972; Huber and Fudenberg, 1968; Inchley, Grey and Uhr, 1970) bind to macrophages and monocytes. This binding is most often due to attachment to the Fc portion of the immunoglobulin molecule (Berken *et al.*, 1966; Henson, 1969; Inchley *et al.*, 1970). SRBC reacted with antiserum collected soon after immunization (Ab1); or purified IgM occasionally bound to a portion of the monocytes, but not to the neutrophils. Some investigators have found that IgM binds to mouse macrophages (Lay and Nussenzweig, 1969) and occasionally to rabbit macrophages (Henson, 1969). Others have failed to find any binding of IgM to macrophages (Berken and Benacerraf, 1966; Inchley *et al.*, 1970) or monocytes (Berken and Benacerraf, 1966; Huber and Douglas, 1970). A minimum of 4000 molecules of IgG on the erythrocytes was necessary for binding to monocytes, which was similar to values previously described (Mantovani, Rabinovitch and Nussenzweig, 1972). However, the minimum number of

antibody molecules on the SRBC for binding or phagocytosis by equine neutrophils was around 12,000. This suggests that the equine monocytes may recognize sensitized particles more readily than neutrophils.

Erythrocytes with IgM and complement attached, readily bound to either monocytes or neutrophils. Complement was indicated in this reaction by elimination of the binding capacity by heating the complement source or reacting fresh serum and sensitized SRBC in the presence of EDTA. This binding was not inhibited by immunoglobulin, but could be blocked by EDTA, indicating the need for Ca^{2+} and/or Mg^{2+} in the attachment reaction. These findings are similar to those previously reported for other species (Henson, 1969; Huber and Douglas, 1970; Lay and Nussenzweig, 1968).

Primary immunodeficiencies of animals have been described only in the nude mice (Pantelouris, 1968), pituitary dwarf mutant mouse strains (Duquesnoy, Kalpaktsoglou, and Good, 1970), cattle (Nansen, 1972) and recently from our laboratory in horses (McGuire *et al.*, 1973). The defect in young horses is associated with an apparent defect in both T- and B-lymphocyte populations. Lymphocytes are almost completely absent in all lymphoid organs, no immunoglobulins are produced, and the animals die within 60 days of birth. The abnormality seems to be transmitted by an autosomal recessive trait. Recent observations show that the lymphocytes present do not respond to mitogens and have little or no surface immunoglobulins. These characteristics suggest that this disease of horses is similar to severe combined immunodeficiency of children.

One possible mechanism of greatly decreased numbers of lymphocytes and the accompanying absences of host defence in animals with CID is a defect in the lymphocyte stem cell (Metcalf and Moore, 1971). In support of this possibility has been the finding that affected children can be reconstituted with cells of the bone marrow (Gatti, Allen, Meuwissen, Hong and Good, 1968), a source of lymphocyte stem cells (Metcalf and Moore, 1971). Because monocytes and neutrophils are thought to arise from the same stem cell as lymphocytes (Metcalf and Moore, 1971; Nowell and Wilson, 1971), a defect in neutrophils and monocytes of animals with lymphocyte dysfunction might be expected. Although the number of lymphocytes of CID horses was greatly reduced or absent, the number of other bone marrow-derived cells was normal. Complement, immunoglobulin, and phytolectin receptors were present on neutrophils and monocytes from affected horses and, by a semi-quantitative method, appeared to be present in normal amounts. Also, no defect was observed in the phagocytic capacity of these cells. Gatti and Good (1972) found that macrophages of two children with severe CID had the ability to assist sensitive lymphocytes in the proliferative response to antigen, suggesting that the macrophages were normal. Douglas and Goldberg (1972) found no receptor abnormalities of the monocytes of patients with acquired agammaglobulinaemia, chronic granulomatous disease, chronic mucocutaneous candidiasis, and Wiskott–Aldrich syndrome. The findings reported here, and by Gatti and Good (1972), suggest that if the abnormality of CID involves the lymphocyte stem cell, then the defect occurs in differentiation of the common stem cell to the specific lymphocyte progenitor.

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