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Studies on the IgA System of the Horse

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Summary. Equine serum and secretions were found to contain a protein which cross-reacted with an antiserum against human IgA, but not with antisera against any other human immunoglobulin. The physicochemical properties of equine IgA resembled those of human IgA. IgA was found to be the immunoglobulin having the highest secretion *vs* serum concentration ratio in equine lacteal and salivary secretions, and to be the protein produced by the majority of immunoglobulin-containing cells in the *lamina propria* of the equine intestine.

INTRODUCTION

It is well established (van der Scheer and Wyckoff, 1940) that horses hyperimmunized with tetanus or diphtheria toxoids produce large amounts of an immunoglobulin called T- or β_2-globulin with an electrophoretic mobility faster than that of slow moving γ-globulin (γ_2). It was first demonstrated by Heidelberger and Pedersen (1937) that high molecular weight antibodies (IgM) existed in sera from horses immunized against pneumococci.

Schwick and Schultze (1960) first outlined that the immunologic relationship existing between the equine T-globulin and the horse's γ_2-globulin resembled the antigenic relationships between human IgA and IgG. It was on this article that Heremans, Vaerman and Vaerman (1963) based their suggestion that the T-globulin was the equine homologue of human IgA.

The complexity of the equine immunoglobulin system was revealed by numerous studies on equine antihapten antibodies (Klinman, Rockey and Karush, 1964, 1965; Rockey, Klinman and Karush, 1964; Klinman, Rockey, Frauenberg and Karush, 1966; Rockey, 1967; Wofsy, Klinman and Karush, 1967; Klinman and Karush, 1967). As a result of these studies, at least six immunoglobulin classes were individualized, namely $IgG_a^{1,2}$ (γG_a), IgG_b (γG_b), IgG_c (γG_c), IgA (= γA or β_{2A}), IgM (= γM or γ_{1M} or 19Sγ), and a 10S γ_1 component. The IgA was generally thought to correspond to the T-globulin on the basis of its high anodal mobility, 7S sedimentation coefficient and high carbohydrate content, but the 10S γ_1 could be related neither to human IgG, IgA or IgM. Several groups of workers have continued to use 'γA' (IgA) and 'T-globulin' as synonyms (Acharya and Rao, 1966; Raynaud, Iscaki and Mangalo, 1965; Hill and Cebra, 1965; Raynaud, Lavergne and Iscaki, 1966; Lavergne, Raynaud and Iscaki, 1966; Fateh-Moghadam, Pichlmayr, Jarosch von Schweder and Knedel, 1967).

Weir and Porter (1966) and Sardesai and Rao (1968) changed the name of IgA to that of IgA(T) on the basis of extensive cross-reactions (involving more than L-chains and perhaps more than the Fab fragment) between the T-globulin and equine slow IgG. Porter's group later used the designation of IgG(T) for the equine T-globulin on the basis of a rather large homology (fourteen out of nineteen aminoacids) between their C-terminal

nonadecapeptides (Weir, Porter and Givol, 1966). This argument led Wofsy, Klinman and Karush (1967) to abandon the term 'IgA' for 'IgG(T)' in their studies on affinity labelling of equine antihapten antibodies and so did Genco, Karush and Tenenhouse (1968) in their report on subunits of polyalanylated γG(T). Similarly, Ernst, Arnon and Sela (1968) employed the terminology of IgG(T) for the T-globulin in their description of cyanogen bromide cleavage of horse immunoglobulins. Besides, Buchowicz, Goch and Zakrzewski (1969) reported that equine IgG and IgG(T) were nearly identical in antigenic properties, amino acid composition, molecular weight, tyrosyl ionization, optical rotatory parameters and accessibility of tyrosyl and tryptophanyl residues to solvents; they differed in carbohydrate content and susceptibility to urea-induced unfolding.

However, Dorrington and Rockey (1968) reverted to the non-committal nomenclature of γT or IgT on the basis of studies using optical rotatory dispersion curves which demonstrated that neither the equine IgT, nor human or canine IgA displayed the Cotton effect at 240 nm which was characteristic of human, rabbit and equine IgG. Moreover, Montgomery, Dorrington and Rockey (1969) pointed out that a smaller number of RNA codon alterations in the C-terminal sequence is required to pass from equine IgG to other mammalian IgGs (rabbit, bovine, human), than is required to change from equine IgG to equine IgT.

Genco, Yecies and Karush (1969) found that equine colostrum and parotid fluid contained IgG_{abc}, IgG(T) and IgM, but little or no secretory IgA resembling the corresponding protein in humans, rabbits and dogs. However, Audibert and Sandor (1968) demonstrated that the T-globulin from equine antitoxic globulins was not the same protein as a fraction which they isolated from equine milk by means of zinc sulphate precipitation and ion exchange chromatography. The latter protein was also found in equine serum but, unlike T-globulin, it did not increase upon hyperimmunization. It was assumed to represent equine IgA.

Recently, Montgomery, Bello and Rockey (1970) reported the similarity of the N-terminal sequence (PCA-Val-Gln-Leu-) of the heavy chains of equine IgG_{ab}, IgT, human IgG_1 Gm(f+), and human IgA_1. It is clear that larger stretches of amino acid sequences need to be unravelled before homologies can be inferred from such arguments.

In 1969, Vaerman, Heremans and Van Kerckhoven found immunological cross-reactions between antisera specific for human alpha chains and a protein present in equine serum and milk. The present report deals with some properties of IgA in equine serum and lacteal secretions, the relative concentrations of IgA in equine colostrum, milk and saliva, and the relative abundance of IgA-containing cells in the equine intestinal mucosa.

MATERIALS AND METHODS

Equine biological fluids

Serum was obtained from several horses at the local slaughter-house.

Equine colostrum and a small sample of equine milk were gifts from Dr L. Podliachouk, Institut Pasteur, Paris, France. A different sample of milk was provided by Dr P. Masson, Department of Experimental Medicine, Louvain. Milk whey was obtained by acidification to pH 4·6 by means of acetic acid and centrifugation of the precipitated casein.

Paired samples of saliva and serum were obtained from four horses (Courtesy Dr F. Dessy, Faculté de Médecine Vétérinaire, Cureghem, Université de Liège, Belgium) after pilocarpine stimulation.

Antisera

These were raised in rabbits by means of footpad injections of the antigens mixed with Freund's complete adjuvant.

Anti-IgA. Rabbit No. 414 was injected with a cross-precipitate obtained by mixing anti-human-IgA ('aA_1': Vaerman *et al.*, 1969) and equine serum. Rabbit No. 434 received purified equine milk IgA (Fraction 2_b from Fig. 5). Antisera were absorbed with Fraction 1 from Fig. 2 and Fractions 3 and 4 from Fig. 5.

Anti-IgM. The antigen was a cross-precipitate obtained by mixing antihuman IgM ('L231': Vaerman and Heremans, 1968) and equine serum. Absorption was carried out with Fractions 2 and 4 from Fig. 3.

Anti-IgG_a. Fraction 1 of the DEAE–cellulose chromatography (Fig. 2) was injected. Absorption was carried out with Fraction 7 from Fig. 2. This antiserum may be designated anti-IgG_b according to a suggestion of Dr J. Rockey (personal communication).

Anti-IgG_c. The antigen was Fraction 4 from Fig. 2. Specificity was achieved by means of absorption with Fraction 1 from Fig. 2.

Anti-IgG(T). This rabbit was injected with the peak protein fraction of a preparative electrophoresis on Pevikon of the proteins from Fraction 6 from Fig. 2, after salting-out with 45 per cent saturated ammonium sulphate. Absorption was carried out by means of Fraction 4 from Fig. 2 and Fractions 1 and 5 from Fig. 3.

Antihorse-light-chains (anti-Ho-L). The light chains were isolated from equine serum IgG_{abc} by means of gel-filtration on Sephadex G-100 in N acetic acid after reduction and alkylation (Fleischman, Porter and Press, 1963). The light chains were injected in the form of alum precipitates.

Fractionation procedures

DEAE–cellulose chromatography. Horse serum (250 ml) was salted out at 40 per cent saturation in ammonium sulphate, pH 6·8, and the centrifuged precipitate was dissolved in and dialysed against 0·005 M Tris-HCl buffer, pH 8·0, before being applied (about 7 g of protein in 100 ml) to a 5 × 40 cm column of DEAE–cellulose equilibrated with the same buffer. Elution proceeded stepwise, using nine successive one litre volumes of (1) 0·005 M Tris-HCl, pH 8·0; (2) 0·01 M; (3) 0·02 M; (4) 0·04 M; (5) 0·04 M Tris-HCl, pH 8·0 + 0·04 M NaCl; (6) id. + 0·08 M NaCl; (7) id. + 0·14 M NaCl; (8) id. + 0·20 M NaCl; and (9) id. + 1·0 M NaCl, respectively. The nine fractions were concentrated to a volume of 25 ml prior to analysis.

Gel-filtration on Sephadex G-200. The eluant was 2 per cent NaCl containing 0·02 M Tris-HCl buffer, pH 8·0, and 0·1 per cent sodium azide. Column lengths of 80–100 cm were used. Gravity flow was maintained under a water pressure of 20 cm. Unconcentrated eluates were tested on semi-quantitative Ouchterlony plates using various specific antisera. The elution diagram (O.D. at 280 nm) was divided into a number of fractions which were concentrated prior to electrophoretic analysis in agarose gels and immunoelectrophoresis.

Zone electrophoresis on Pevikon. Ten millilitres of twice-concentrated equine serum were applied to a 30 × 50 × 1 cm block of Pevikon. Electrophoresis proceeded for 36 hours at 4° in sodium barbiturate buffer, pH 8·4, under a voltage gradient of 7 V/cm. The block was cut up into forty 1 cm wide fractions which were eluted each in 20 ml of saline. Each fraction was analysed for total protein content (Lowry, Rosebrough, Farr and Randall, 1951) and for its content of equine immunoglobulins by semi-quantitative Ouchterlony plates using specific antisera.

Analytical procedures

Agarose gel electrophoresis was performed according to Laurell (1965); immunoelectrophoresis and Ouchterlony analyses following established procedures; single radial immunodiffusion according to Mancini, Carbonara and Heremans (1965); and starch gel immunoelectrophoresis according to Poulik (1963).

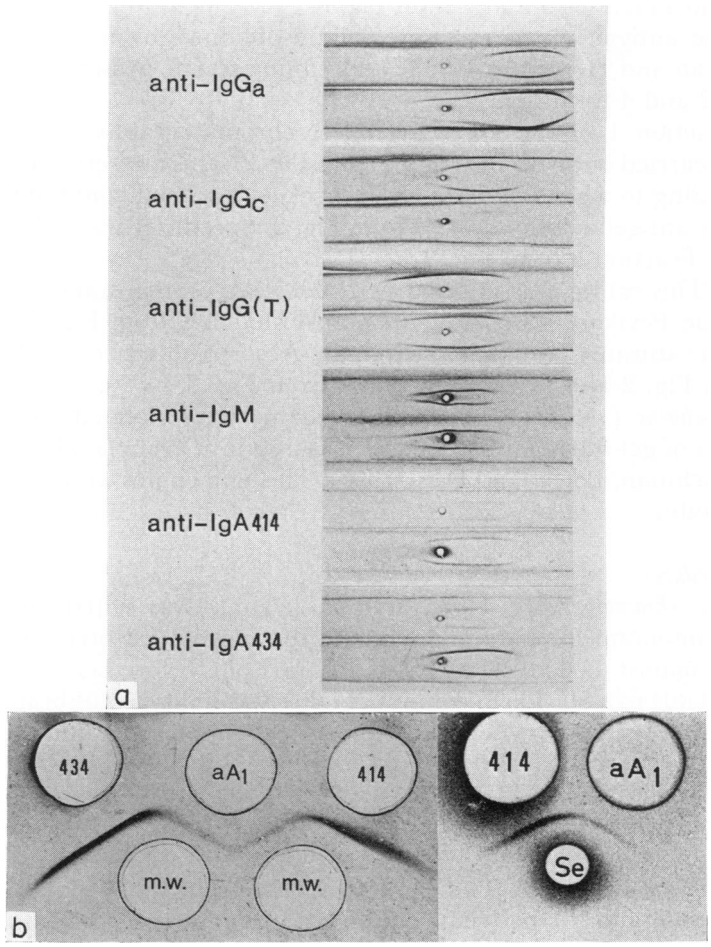

FIG. 1. Immunological analyses of equine serum and milk proteins. (a) Immunoelectrophoreses. In each of the six plates, equine serum (upper well) and colostrum (lower well) are developed with specific antisera against equine immunoglobulins. Anode to the left. (b) Comparative Ouchterlony analyses. Antihuman-IgA(aA_1) and antiequine-IgA (No. 414 and 434) diffuse towards equine serum (Se) and milk whey (m.w.).

Immunohistochemical techniques

Pieces of intestinal mucosa, spleen and lymph nodes from two horses were snap-frozen with dry ice less than 1 hour after death. Sections (4–6 μ thick) were dried in air, fixed in methanol, washed in phosphate buffered saline and incubated for 30–60 minutes with various fluorescein-labelled antisera specific for equine immunoglobulins. After three

washes in buffered saline, the sections were mounted in buffered glycerol prior to examination with the UV microscope (Crabbé, Carbonara and Heremans, 1965).

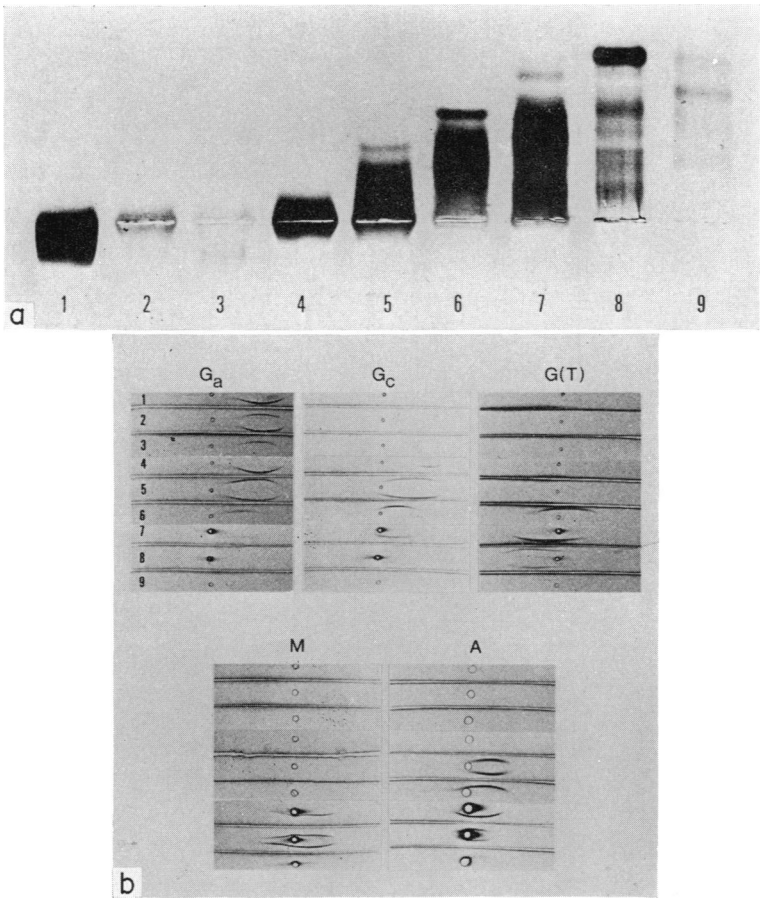

FIG. 2. DEAE-cellulose chromatography of equine serum. (a) Agarose gel electrophoresis of DEAF-Fractions 1 through 9 (see Materials and Methods). Anode upwards. (b) Immunoelectrophoreses of DEAE-Fractions 1 through 9 revealed by specific antisera against equine immunoglobulins G_a, G_c, G(T), M and A. Anode to the left.

RESULTS

The specificity of the various antisera against equine immunoglobulins is illustrated by the immunoelectrophoreses of whole equine serum and colostrum (Fig. 1a). In order to confirm that the antiequine-IgA antisera (No. 414 and No. 434) truly reacted with the equine protein homologue of human IgA, these antisera and the antihuman-IgA antiserum aA_1, which cross-reacted with the horse, were allowed to diffuse, side by side, against equine serum or milk whey (Fig. 1b). Complete fusion of the precipitin lines was observed, demonstrating that all the anti-IgA antisera reacted with the same protein, i.e. equine IgA.

To compare equine serum IgA to human serum IgA and other equine immunoglobulins, equine serum was fractionated by DEAE-cellulose chromatography, Sephadex G-200 gel-filtration and zone electrophoresis on Pevikon.

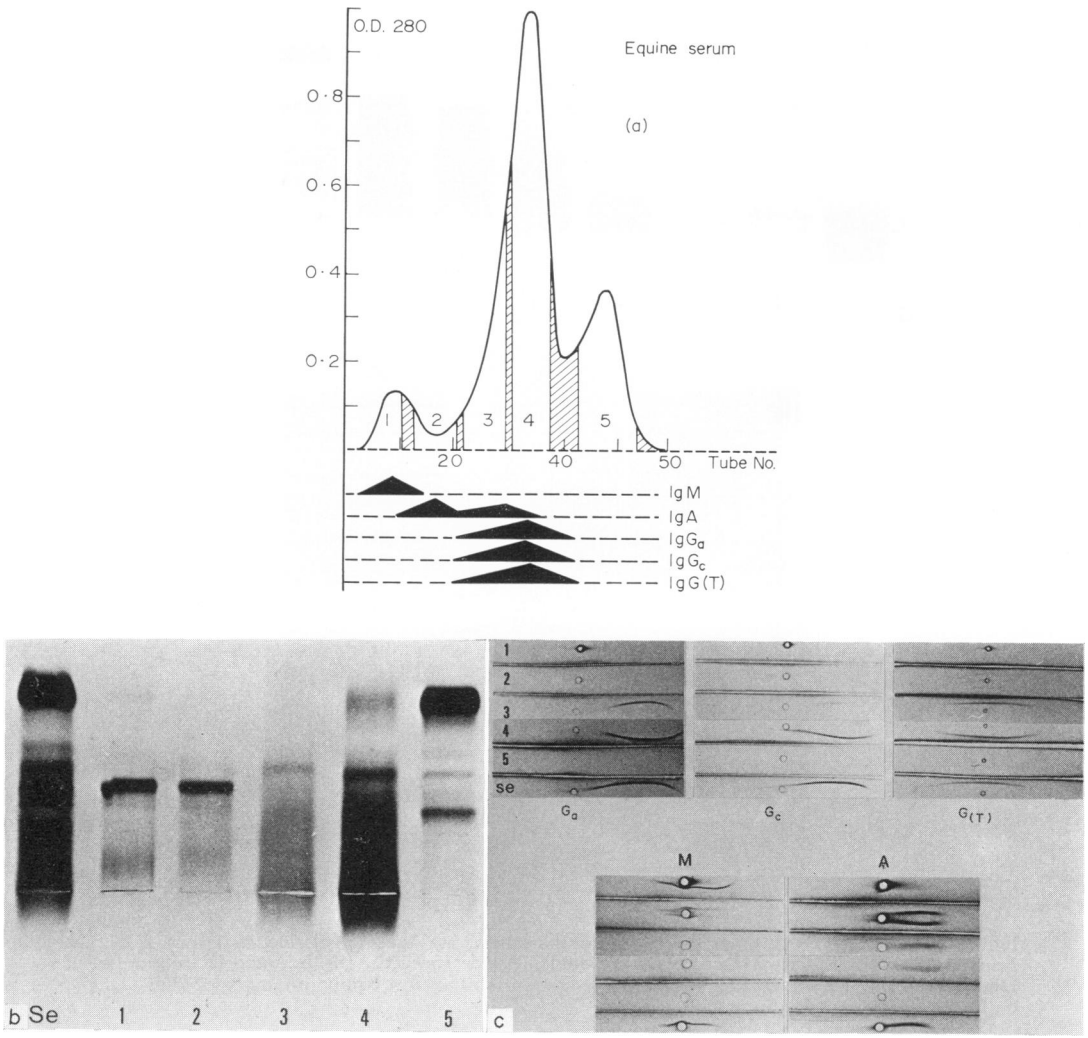

FIG. 3. Gel-filtration of equine serum on Sephadex G-200. (a) Elution pattern and semiquantitative immunochemical localisation of equine immunoglobulins. (b) Agarose gel electrophoresis of Sephadex-Fractions 1 through 5 compared to equine serum (Se). Anode upwards. (c) Immunoelectrophoreses of Sephadex-Fractions 1 through 5 compared to equine serum (Se), developed by antisera specific for equine immunoglobulins G_a, G_c, G(T), M and A. Anode to the left

On DEAE-cellulose, equine serum IgA was eluted mainly in Fractions 5, 6, 7 and 8, with traces in Fraction 9 (Fig. 2a, b). Its elution started well after that of IgG_a, slightly after that of IgG_c, slightly before that of IgG(T) and well before that of IgM.

Upon gel-filtration on Sephadex G-200 (Fig. 3a–c), equine serum IgA displayed a bimodal distribution, with the first peak being eluted immediately behind the IgM peak,

while the second peak eluted just before that of the various equine IgGs. The relative importance of these two IgA peaks varied somewhat from horse to horse, but the first peak always represented an important fraction of equine serum IgA as determined by quantitative immunodiffusion.

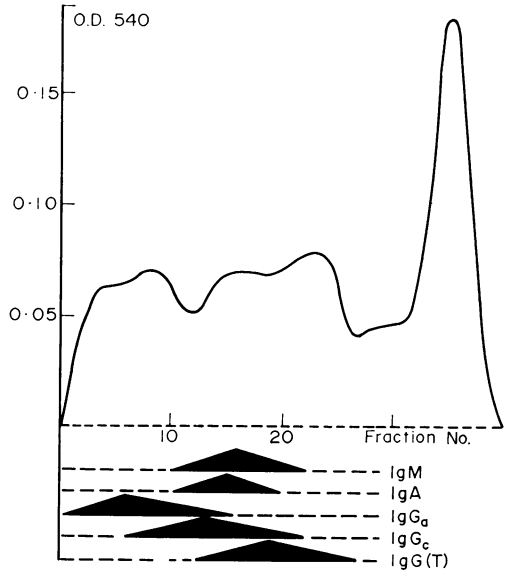

FIG. 4. Electrophoresis on Pevikon of equine serum. Electrophoretic pattern (anode to the right) and immunochemical localisation of equine immunoglobulins G_a, G_c, G(T), M and A.

TABLE 1

COMPARATIVE CONCENTRATIONS OF VARIOUS IMMUNOGLOBULINS IN EQUINE SECRETIONS AND SERUM (SECRETION: SERUM CONCENTRATION RATIOS)

	IgA	IgM	IgG_a	IgG_c	IgG(T)
Saliva 1	0·107	<0·0029	0·0003	<0·0002	0·0002
2	0·0142	<0·0012	0·0004	0·0003	0·0002
3	0·105	<0·0057	0·0001	0·0005	0·0001
4	0·074	0·0023	0·0012	0·0001	0·0009
Colostrum*	7·115	4·504	6·963	0·205	1·854
Milk*	0·321	0·029	0·021	0·0013	0·011

* Compared to a pool of normal equine serum.

When analysed by Pevikon electrophoresis (Fig. 4), equine serum IgA displayed an heterogeneous electrophoretic mobility, slower than that of IgG(T), about the same as that of IgM, but distinctly faster than that of IgG_a and IgG_c.

It can be inferred from Fig. 1 that equine colostrum is very rich in IgG_a, poor in IgG_c, relatively rich in IgG(T), but also very rich in IgM and especially in IgA, as compared to equine serum. Quantitative ratios of immunoglobulin concentrations, in secretions *vs* serum, were established by single radial immunodiffusion in four equine salivas, one sample of milk, and one of colostrum. The results are listed in Table 1. IgA displayed the highest secretion *vs* serum concentration ratio in all samples. All equine immunoglobulins had much higher ratios in colostrum than in milk.

When mare's milk whey was submitted to Sephadex G-200 gel-filtration (Fig. 5a–c), IgA was localized to the first two fractions, particularly the second one. When the latter fraction was refiltered on G-200, the second half of its eluates (Fraction 2_b) appeared to consist entirely of IgA. The various equine IgGs were all eluted in Fraction 3 which was almost devoid of IgA. Fractions 4–7 were devoid of immunoglobulins. Fraction 4 was pink

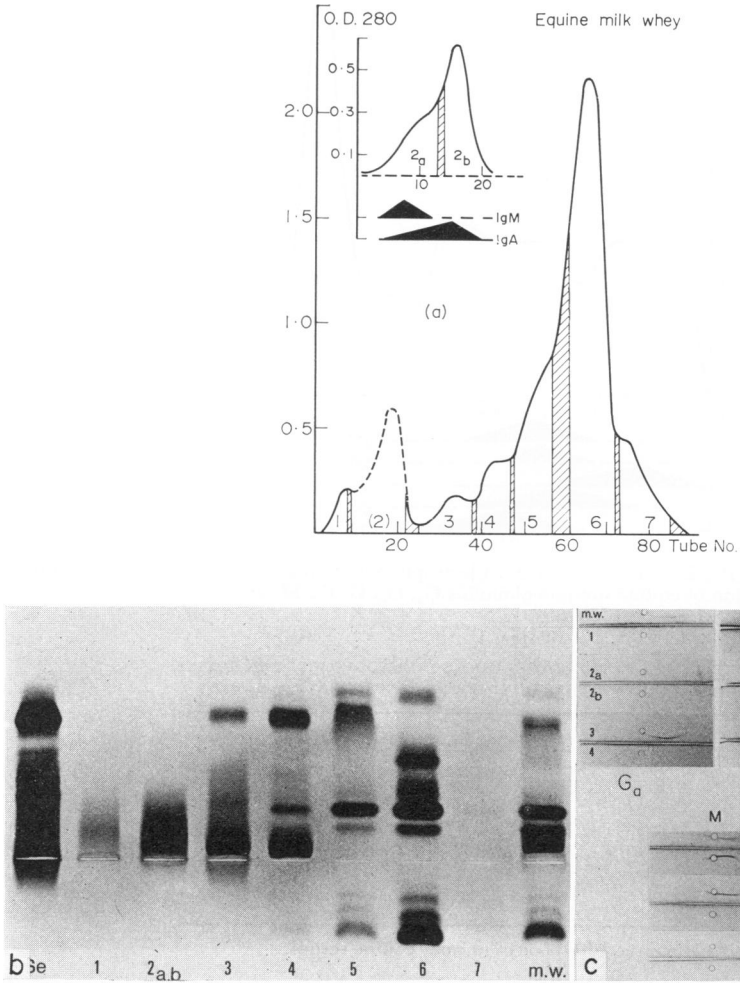

FIG. 5. Gel-filtration of equine milk whey on Sephadex G-200. (a) Elution pattern. The elution pattern and immunochemical localisation of equine IgM and IgA along successive eluates of a second gel-filtration on concentrated Fraction (2) is inserted. (b) Agarose gel electrophoresis of concentrated Sephadex-Fractions 1 through 7, compared to equine milk whey (m.w.) and serum (Se). Anode upwards. (c) Immunoelectrophoreses of concentrated Sephadex-Fractions 1, 2a, 2b, 3 and 4, revealed by antisera specific for equine immunoglobulins G_a, G_c, G(T), M and A. Anode to the left.

and Fraction 6 displayed 3 cathodal components. The pink fraction may contain the equine homologue of human lactoferrin, and one of the cathodal components of Fraction 6 may be lysozyme.

Equine milk IgA (Fraction 2_b from Fig. 5), when submitted to immunoelectrophoresis in acid-urea starch-gel showed two cathodal, fast moving, components which reacted (Fig. 6)

with an antiserum against equine $IgG_{a,b,c}$ light chains. Since this result was obtained on a sample that had not been submitted to any disulphide-splitting procedure, it must be concluded that at least some molecules of equine IgA have their light-chains not covalently linked to heavy chains.

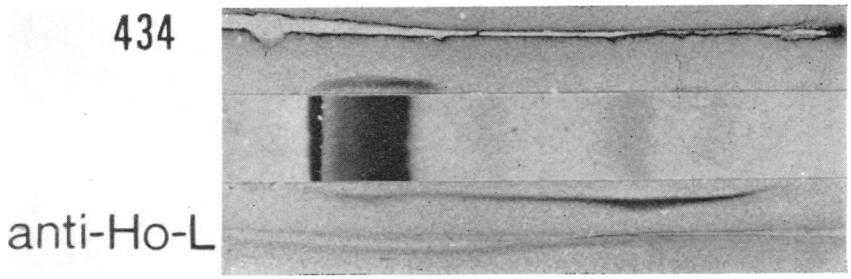

FIG. 6. Acid-urea starch-gel immunoelectrophoresis of unreduced IgA from equine milk whey. No. 434, anti-equine IgA. Anti-Ho-L, antihorse-light chains. Cathode to the right.

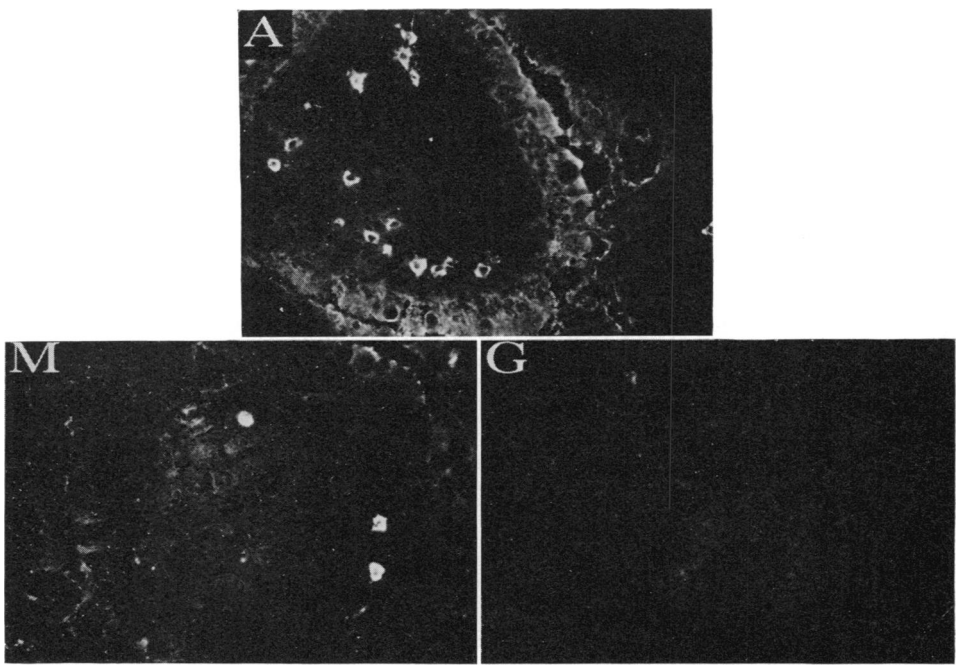

FIG. 7. Cryostat sections of equine duodenal mucosa. Incubation with antisera specific for equine immunoglobulins A, M and G_a (G). Sections incubated with anti-IgG_c or anti-IgG(T) gave patterns similar to that obtained with anti-IgG_a. × 125.

When equine serum and milk were tested in Ouchterlony plates against two different antisera against equine milk IgA, no antigenic determinants specific of equine milk IgA, and absent from serum IgA, could be demonstrated.

In the intestinal mucosa, IgA-containing cells were definitely more abundant than

IgM- or IgG-containing cells (Fig. 7). However, the absolute numbers of immunoglobulin-containing cells seemed rather small compared to the abundance of IgA cells found in the intestinal mucosae of the human (Crabbé *et al.*, 1965), rabbit (Crandall, Cebra and Crandall, 1967), mouse (Crabbé, Bazin, Eyssen and Heremans, 1968), dog (Vaerman and Heremans, 1969a), rat (Nash, Vaerman, Bazin and Heremans, 1969), pig (Porter and Allen, 1970; Vaerman, 1970), and cat (Vaerman, 1970). The majority of these IgA-type cells were seen in upper parts of the bowel (duodenum and proximal jejunum). The deeper contents of the crypts also stained specifically for IgA and to a lesser extent for IgM. In contrast, the spleen and lymph nodes examined were very poor in IgA-type cells, but contained various proportions of IgM- and IgG-cells. The spleen was particularly rich in IgM-containing cells.

DISCUSSION

Although amino acid sequences of equine and human alpha chains have not been compared so far, the existence of a protein called equine IgA can reasonably be inferred from the data presented in this study. In our opinion, the decisive criterion resides in the cross-reaction demonstrated between the antihuman-IgA antiserum and equine serum and milk IgA. Equine IgA resembles its counterpart in the human by more than one feature: (i) the predominance of IgA-type cells in the intestinal mucosae; (i) its high secretion *vs* serum concentration ratio; (iii) the occurrence of IgA molecules whose light chains are not disulphide-linked to the heavy chains, thus resembling IgA_2-subclass molecules described in the human and murine species; and (iv) the distinct properties of IgA in equine serum and milk upon various fractionation procedures. The secretory component of equine exocrine IgA has so far not been demonstrated, but only two antisera were tested.

It was interesting that an important fraction of equine serum IgA had a molecular size larger than that of the 7S monomer and smaller than IgM, as judged from the gel-filtration on Sephadex G-200 experiment. Montgomery *et al.* (1969) had already suggested that the 10S γ_1 anti-Lac antibody and the related serum protein, first described by Rockey (1967), might be the equine equivalent of human IgA. The present data evidently confirm this assumption. In this connexion, it might be outlined that so far, only the human (Heremans, Heremans and Schultze, 1959), the hamster (Bienenstock, 1970), and perhaps the rat (Bistany and Tomasi, 1970; Stechschulte and Austen, 1970) have been shown to possess serum IgA with a majority of 7S monomer molecules, in contrast to the dog (Johnson and Vaughan, 1967; Vaerman and Heremans, 1968; Vaerman and Heremans, 1969b), the cat (Vaerman, 1970), the pig (Bourne, 1969; Vaerman and Heremans, 1970a), the cow, goat and sheep (Vaerman, 1970; Pahud and Mach, 1970; Mach and Pahud, 1971), the mouse (Nash, Vaerman, Bazin and Heremans, 1970; Grey, Sher and Shalitin, 1970), and the hedgehog (Vaerman, 1970) which are all species in which a large fraction of serum IgA exists in the form of a polymer—probably a dimer—smaller than 19S IgM.

In equine colostrum, IgA is a rather minor component in absolute amounts, whereas in equine milk, IgA, represents the predominant immunoglobulin, IgM and the various IgG subclasses being present only in small amounts (Figs 1 and 5). A similar situation prevails in the dog (Vaerman and Heremans, 1969b) and sow (Porter, Noakes and Allen, 1970; Vaerman and Heremans, 1970b).

It is thus obvious that in the horse, as in other mammals, there exists an IgA system analogous to that of the human.

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