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Immunoglobulins A, G, and M in Serum and in Some Secretions of Monkeys (*Macaca fascicularis* syn. *irus*)

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The purpose of this investigation was to study the distribution and levels of the following immunoglobulins, IgA, IgG, and IgM, in sera and in some secretions of monkeys (*M. fascicularis*). IgG, IgA, and IgM were isolated from monkey serum and secretory IgA was separated from monkey milk by combined gel filtration and ion-exchange chromatography. These pure preparations served as standards to quantitate immunoglobulins in sera and secretions by single radial immunodiffusion. Antisera were raised in the rabbit against the pure immunoglobulins and also against the whole secretions to identify the immunoglobulins in immunoelectrophoresis. In common with humans, the major immunoglobulin in serum and amniotic fluid is IgG and the IgG/IgA ratio is greater than unity. In secretions IgA is the dominant immunoglobulin and the IgG/IgA ratio is less than 1. In general, the levels of immunoglobulins in the sera and secretions of monkeys paralleled the levels found in humans. No age-related increase in immunoglobulin levels was detected in the sera of monkeys.

Nonhuman primates have been used as experimental animals for a wide variety of biomedical investigations. Their close similarity to humans, both anatomical and physiological, has made it possible to extrapolate from the monkey to the human situation with far more certainty than with other animal models.

Despite the great popularity of nonhuman primates in the study of noninfectious immunology and organ and tissue transplantation, there is relatively little known about the basic immunology of these animals. The rhesus monkey (*Macaca mulatta*) and the so-called crab-eating macaque (*M. fascicularis* syn. *irus*) are among the most popular species used in research, and most data accrue from studies of these primates. Much work has been concerned with immunotaxonomy as it relates to phylogeny, but there are few data on the distribution and quantitation of the immunoglobulin (Ig) classes (IgG, IgA, IgM, IgD, and IgE). Only scattered data are available on the normal levels of these immunoglobulins in the sera of primates (12, 17, 20, 24). IgG, IgA, and IgM are the major classes in serum, with IgD and IgE present at low concentrations; however, virtually nothing is reported on the levels of immunoglobulin in other body fluids.

The purpose of the present investigation was to study the distribution and levels of IgA, IgM, and IgG in the sera and in some of the body fluids of monkeys (*M. fascicularis*). It was hoped that the data would provide more comprehensive background information on the species and perhaps furnish a better understanding of the possible mechanisms of immunological protection at mucosal surfaces. The identification and quantitation of immunoglobulins present in the oral cavity may help to clarify the way in which dental caries and periodontal disease could be controlled by vaccination procedures.

MATERIALS AND METHODS

Animals. Monkeys (*M. fascicularis*) maintained in the primate colony of the Department of Dental Science of the Royal College of Surgeons of England were used in this study.

Tranquilization. Unless otherwise stated, all animals were tranquilized by intramuscular injection of phencyclidine hydrochloride (Sernylan, Parke-Davis), 2 mg/kg of body weight. Samples were usually collected 17 h after the last intake of food. No samples were collected from animals taking part in vaccination experiments, because repeated immunization could lead to raised immunoglobulin levels.

Collection of body fluids. (i) Blood. Venous blood was collected from the femoral vein of tranquilized animals. Serum was harvested by centrifugation and frozen in small aliquots. Pooled serum samples to be separated by column chromatography were subjected to ammonium sulfate fractionation to iso-

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late the gamma globulins. Sufficient saturated ammonium sulfate solution was added to the serum to give a final concentration of 35% (15). Precipitation was repeated three times.

(ii) Whole saliva. A concomitant effect of phenycyclidine hydrochloride tranquilization was to increase salivation, and it was possible to aspirate whole saliva from the mouth with a Pasteur pipette. Samples were discarded if they appeared to be contaminated with blood. After collection, the saliva was centrifuged free of debris and cells and frozen until required.

(iii) Parotid and submandibular saliva. Parotid and submandibular saliva was collected by cannulating the salivary ducts under general anesthesia. Large male or female animals were induced with sodium methohexitone (Brietal, Eli Lilly), 20 mg/kg of body weight, and a 2- to 3-mm endotracheal tube was passed intranasally after spraying the vocal cords with 2% lignocaine (Xylocaine, Astra Hewlett). Anesthesia was maintained with 1% halothane (Fluothane, ICI), nitrous oxide (2 to 3 liters/min), and oxygen (1 liter/min). The airway was protected with a gauze throat pack. The parotid ducts were identified and dilated with either tapered glass rods or ball-ended glass explorers. A beveled polyethylene tube was then introduced into the duct. The submandibular ducts were cannulated by grasping the sublingual papilla with fine dissecting forceps, enlarging the duct with a fine wire, and introducing a fine, beveled polyethylene tube. In one animal it was possible to cannulate the sublingual ducts, but this was not attempted routinely. Salivary flow was stimulated by subcutaneous injection of pilocarpine nitrate (1 mg/kg of body weight).

(iv) Lacrimal fluid, nasal fluid, and sweat. It was possible to collect lacrimal fluid, nasal fluid, and sweat under general anesthesia once pilocarpine had been administered. Lacrimal fluid was aspirated from the inner canthus of the eye with a Pasteur pipette. Sweat was aspirated from the palms of the hands and nasal fluid from the nasal cavity by this method.

(v) Milk. Breast milk was expressed from the nipple by gently massaging the breast. Milk samples to be used for column chromatography were defatted by centrifugation at 10,000 × *g* for 1 h at +4 C. Casein was precipitated by lowering the pH to 4.6 by the dropwise addition of 0.1 N HCl, and the supernatant was cleared by centrifuging at 12,000 × *g* for 0.5 h (7).

(vi) Amniotic fluid. Amniotic fluid was collected from pregnant female animals undergoing hysterectomy or Caesarean section. In either case the amnion or chorioamniotic membrane was pierced with a fine sterile needle and fluid was aspirated into a syringe. The amniotic fluid was centrifuged free of cells and stored frozen.

(vii) Column chromatography. Immunoglobulins were isolated from serum and milk by combined gel filtration and ion-exchange chromatography. Body fluids were initially fractionated by gel filtration using Sephadex-G200 equilibrated with 0.1 M phosphate buffer (pH 6.8) in a Sephadex-K 15/90 column (Pharmacia GB Ltd). Elution was effected using the same equilibration buffer. Protein concentration in the eluate was monitored at 280 nm with an ultraviolet absorptiometer (LKB). The presence of immunoglobulins in the eluate was identified by setting up the fractions in gel diffusion against specific rabbit antihuman IgG, IgA, and IgM immunoglobulin reagent (Miles Seravac). Fractions of the serum and milk containing immunoglobulins were subjected to further purification by ion-exchange chromatography using diethylaminoethyl-cellulose (Whatman DE52) equilibrated with 0.01 M phosphate buffer, pH 7.5. Elution was effected by increasing ionic strength. After three column volumes of starting buffer, the following molarities of sodium chloride were added in a stepwise sequence: 0.05, 0.1, 0.2, 0.3, 0.4, and 1.0 (7). Final purification was achieved by further Sephadex-G200 filtration until symmetrical peaks were obtained. The purity of the immunoglobulins was tested in immunoelectrophoresis against rabbit anti-whole monkey serum and rabbit anti-monkey milk serum.

(vii) Gel diffusion. Double diffusion (25) was carried out using 1% (wt/vol) agar (Ionagar no. 1, Oxoid) in phosphate-buffered saline. Diffusion was allowed to proceed for 48 h at 4 C in a diffusion chamber.

(ix) Immunoelectrophoresis. Immunoelectrophoresis was carried out on a microscale (28), using 1% agar (Ionagar no. 1, Oxoid) in 0.1 M barbitone acetate buffer, pH 8.6. Electrophoresis was conducted at a constant current of 6 mA/inch (width) (2.54 cm) of gel for 2 h at 4 C.

(x) Immunoglobulin quantitation. Measurement of the levels of immunoglobulin were made using single radial immunodiffusion (22). Commercially prepared anti-human immunoglobulin diffusion plates were used (Immuno-plate, Hyland Laboratories Ltd.). Plates for two ranges of concentration were used: a standard plate and a low-level plate. Samples of monkey body fluids were concentrated by freeze-drying where necessary to bring them into the range of the immunodiffusion plates supplied. Chromatographically pure monkey immunoglobulin standards were included in every run.

(xi) Production of antisera. Antisera to the major monkey immunoglobulins and various body fluids were raised in the rabbit. Light-chain cross-reactivity was removed from the antisera raised against the major monkey immunoglobulin classes by incubating the antisera at 37 C overnight with monkey immunoglobulin classes against which the antisera was not directed. The absorbed antisera were checked by immunoelectrophoresis.

RESULTS

Production of antisera. The anti-immunoglobulin antisera raised in rabbits all showed some slight light-chain cross-reactivity, which was subsequently removed by absorption. The IgA isolated from monkey milk eluted close to the void volume of Sephadex-G200, which suggested that it was secretory IgA. However, rabbit antisera raised against the immunoglobulin

failed to produce a secretory component spur in immunoelectrophoresis. Whole antisera against the monkey secretions were successfully raised in all cases except with urine. Even after repeated immunization with concentrated urine only a single, very faint anionic arc was ever detected. Insufficient nasal secretion, lacrimal secretion, sublingual saliva, and sweat were available to allow antisera to be produced. In a number of secretions immunoglobulins detected by single radial immunodiffusion and double diffusion could not be detected by immunoelectrophoresis.

Quantitation of immunoglobulins by single radial immunodiffusion. The levels of immunoglobulins detected in serum and secretion are summarized in Tables 1 and 2. The concentrations of immunoglobulins in the monkey serum samples determined with the monkey immunoglobulin standards agreed fairly well with the levels estimated using human standards and human antisera, as might have been expected by the high level of cross-reactivity between the monkey and human proteins (36). However, in contrast to Wang et al. (36), the quantitation of both IgG and IgM agreed much more closely than did the measurement of IgA. This may, in part, have resulted from the production of very faint precipitin lines that invariably required intensification when IgA was measured. When the monkey secretory IgA standards were referred to standard curves produced with monkey serum IgA standards, the measured concentrations were underestimated by a factor of approximately three, and this is consistent with the findings reported by Tomasi and Bienenstock (31) for human proteins. The IgG/IgA ratios calculated for monkey serum (6.6:1) and amniotic fluid (2.7:1) are characteristic of internal secretions, where this ratio is greater than one. In contrast, all the external secretions investigated had an IgG/IgA ratio of less than unity (Table 1).

DISCUSSION

The levels of immunoglobulins detected in serum and secretions of *M. fascicularis* in general compare closely with those reported in humans. In common with human studies the range of values is wide, and more accurate mean values would require measurements to be made on much larger samples. In the case of many of the primate secretions studied, the practical difficulties involved in obtaining samples have necessarily restricted the number of samples available for study. The use of monkey immunoglobulin standards has gone some way towards eliminating any error introduced by assaying monkey proteins with anti-human antisera, despite the considerable degree of cross-reactivity (36). Tomasi and Bienenstock (31) have drawn attention to the problems of quantitating immunoglobulins in secretions. Radial immunodiffusion, probably the most commonly used method of immunoglobulin quantitation, is not sufficiently sensitive to be used on native unconcentrated secretions, and preliminary concentration is almost always necessary. How-

TABLE 1. *Immunoglobulin concentrations in serum and secretions*

Sample (no. of samples)	Concn (mg/100 ml, mean and limit values)			Ratio	
	IgA	IgG	IgM	IgG/IgA	IgG/IgM
Serum (60)	233	1,539	133	6.6	11.6
	80–470	800–1,900	34–250		
Amniotic fluid (10)	28.4	67.1	2.5	2.37	2.69
	0–101.0	0–250.0	0–13.0		
Milk (13)	241.60	4.38	5.30	0.018	0.83
	33.0–462.0	0–8.0	0–14.0		
Urine (5)	0.27	0.13		0.48	
	0–0.69	0–0.43			
Nasal secretion (7)	294.42	94.6	10.6	0.32	8.9
	33.0–387.0	14.0–164.0	3.2–13.0		
Lacrimal secretion (7)	9.15	2.20		0.24	
	7.1–10.2	0–4.3			
Sweat (1)	0.33	0.10		0.30	
Whole saliva (unstimulated) (30)	36.42	4.14	0.76	0.11	5.43
	6.0–66.0	0.75–11.50	0–3.25		
Parotid saliva (stimulated) (10)	3.60	0.54		0.15	
	2.9–4.1	0.36–0.94			
Submandibular saliva (stimulated) (10)	8.07	0.63		0.08	
	4.3–15.1	0.30–0.94			
Sublingual saliva (stimulated) (1)	4.80	0.66		0.14	

TABLE 2. *Serum immunoglobulin levels related to age*

Immunoglobulin	mg/100 ml (mean and limit values)[a]					
	1-2[b]	3-4	5-6	7-8	9-10	10+
IgA	240 80-410	289 190-470	257 150-450	252 125-470	264 180-350	297 160-350
IgG	1,487 1,200-1,900	1,591 1,300-1,900	1,469 1,275-1,800	1,458 980-1,650	1,628 800-1,900	1,600 1,450-1,900
IgM	100 34-150	96 44-150	173 86-250	152 75-250	111 58-220	167 112-250

[a] Ten samples tested in each case.
[b] Age of animals in years.

ever, concentration procedures may result in considerable loss and possible denaturation of the proteins. Of the immunoglobulins, secretory IgA is probably the most resistant to denaturation, because this is a property necessary for the immunoglobulin to function effectively at mucosal surfaces (4). The quantitation of IgG and IgM in secretions can be complicated by the presence of breakdown products of these immunoglobulins, which may still precipitate with antisera so that the level estimated probably only represents the maximum possible level to be found in that particular secretion. This is especially so in urine, where components of gamma globulins have been detected one-fifth to one-sixth the size found in serum with sedimentation coefficients as low as 1.6*S* (13).

The levels of IgG, IgA, and IgM determined in sera from *M. fascicularis* are similar to serum immunoglobulin levels reported in human subjects (1, 4, 6, 18, 29, 30). However, a clear relationship between age and serum IgA levels evident in humans was not apparent in monkeys (Table 2). This may reflect the relative rapidity with which monkeys attain maturity in comparison to humans. Alternatively, the small sample size, coupled with measurements made at fairly large age increments, may have masked any incremental increase in serum IgA levels. However, there is some indication that age-related increases in serum IgA and IgM may be detected in chimpanzees (34) and in rhesus monkeys (35). The serum immunoglobulin levels found in *M. fascicularis* are similar to those reported (23) for the rhesus monkey (*M. mulatta*); however, the serum IgA levels were markedly higher than those reported in a small group of rhesus monkeys by Houba et al. (16). It is interesting that Goodman and Poulik (14) found that sera from cynomolgus monkeys contained 17.2% gamma globulin, and this was significantly higher than a level of 10.7% found in rhesus monkeys.

IgG and IgA have been detected in human amniotic fluid (9, 11), but the levels were considerably lower than those determined in monkeys. In addition, low levels of IgM were detected. It is not known whether the immunoglobulins were derived from mother or fetus, although studies of IgG in human amniotic fluid (8) suggest a maternal origin.

It is well known that colostrum is an important source of protective immunoglobulin for the neonate in a number of species of animals. High levels of secretory IgA have been reported in human colostrum (5, 9), and a ratio of IgA to IgG in excess of 100 has been reported (5). The concentrations of immunoglobulins found in breast milk from monkeys are considerably lower than the colostral values reported in human samples. However, few, if any, of the monkey milk samples were true colostrum, and the values determined reflect those more characteristic of milk collected later after parturition.

The detection of IgA and IgG in monkey urine is consistent with the findings of Turner and Rowe (33) and Bienenstock and Tomasi (2) in humans. Tracings obtained after filtering monkey urine through Sephadex-G200 closely resemble those described (33) when concentrated human urine was similarly chromatographed. Close agreement of immunoglobulin concentration is achieved if the 24-h levels reported by Bienenstock and Tomasi (2) are expressed in milligrams per 100 milliliters.

Remington et al. (26) showed that IgA was the major immunoglobulin present in nasal washings and that IgG was present in trace amounts but no direct quantitation was undertaken. It was demonstrated that, whereas only two precipitin lines were normally detected in immunoelectrophoresis against nasal washings, the number of lines increased dramatically when the nasal mucosa was irritated, resulting in subsequent serum leakage. It is possible that the high levels of immunoglobulins reported in the monkey specimens were the result of serum leakage caused by trauma to

the nasal mucosa when the samples were collected with a Pasteur pipette.

McLellan et al. (21) have reviewed the results of studies in which immunoglobulins have been measured in human lacrimal fluid. In almost every investigation IgA has been shown to be the dominant immunoglobulin class, whereas IgG has been detected in low or trace amounts. IgM is not usually detectable, but it has been demonstrated (19) that mild irritation to the eye, by rubbing, for example, can introduce serum proteins not normally found in tears. It is likely that the introduction of absorbent materials to collect samples of tears has the same effect and could explain the detection of IgM in some studies.

The presence and characterization of immunoglobulins in saliva has attracted considerable attention in recent years because of their possible role in immunity to dental caries. Chodirker and Tomasi (9) found that, in common with other external secretions, the IgG/IgA ratio in parotid saliva was less than unity. Abnormally high levels of IgA were reported, especially when it is considered that serum standards were used. These values are at variance with the much lower levels determined in parotid saliva (32), although 7S serum IgA standards were also used. Claman et al. (10) reported a median concentration of IgA of 9.5 mg/100 ml in normal human parotid saliva, and this value agrees well with that estimated by Salvaggio et al. (27). Claman et al. (10) detected IgG in a single sample of parotid saliva only and IgM was never detected. Little differences was found in the concentration of IgA from the right and left glands. In a extensive and detailed study, Brandtzaeg et al. (5) measured the levels of IgA, IgG, and IgM in unstimulated and stimulated parotid and submaxillary saliva and in unstimulated whole saliva. Only IgA could be detected in the unstimulated parotid and submaxillary saliva, but IgA, IgG, and IgM were all detected in stimulated samples. Brandtzaeg (3) showed that, although immunoglobulin transfer increased 2.5 times when the flow of parotid saliva was stimulated, this was insufficient to compete with the increased flow rate. As a result, there was a fourfold reduction of IgA concentration in stimulated as compared with unstimulated parotid saliva. The concentrations of immunoglobulins in combined submandibular and sublingual saliva appear to approximate the concentrations detected in parotid saliva (5, 10).

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