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IRON-BINDING PROTEINS IN VITREOUS HUMOUR

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The soluble protein composition of Macaque monkey vitreous humour was studied in order to understand its iron-binding properties. The protein content of vitreous humour was 217 μg/ml ± 4.6%, 40% of which was serum albumin and 30% an iron-binding protein of hydrodynamic properties identical to that of transferrin or lactoferrin. Relative to serum, the vitreous humour contained a 13-fold excess of this protein(s). Isoelectric focusing, iron-binding and immunoelectrophoretic studies indicated that both vitreous humour and aqueous humour contained lactoferrin as well as serum transferrin. The iron-binding capacity of these proteins in vitreous humour was equivalent to the mass of haemoglobin iron contained in at least 570000 monkey erythrocytes. It was concluded that the intraocular lactoferrin originated from within the eye. These iron-binding proteins may play a protective role in ocular disturbances such as vitreous haemorrhage, iron foreign body toxicity and infection.

Introduction

The composition and origin of soluble proteins in aqueous and vitreous humours are still uncertain. It has been suggested that most of these proteins originate in serum and that they enter aqueous and vitreous via blood ocular barriers [1–5]. Immunochemical studies have revealed that there are a number of vitreous proteins which are not found in serum [3,4,6]. Thus, it would appear that the soluble proteins of aqueous and vitreous humours originate not only in serum but also from within the eye itself.

It has been reported that the serum glycoprotein, transferrin, is a major constituent of rabbit vitreous humour [5]. This observation has not been confirmed and nothing is known of the iron-binding properties or the biological function of transferrin in vitreous humour. The purpose of the present work was to examine the soluble proteins of vitreous humour by methods more definitive than used previously and to establish the presence and characteristics of transferrin in vitreous humour from species other than the rabbit. We describe for the first time the presence of lactoferrin as a major iron-binding protein in aqueous and vitreous humour and confirm the presence of transferrin in these fluids. Our finding of a high latent iron-binding capacity of these proteins suggests that they may play an important protective role in the eye.

Materials and Methods

Animal studies. Young adult Rhesus monkeys (*Cynomolgus fasicularis*, body weight 4–6 kg) were used for all experiments. Examination of the eyes and sampling of aqueous and vitreous humours were carried out under general anaesthesia, using a combination of intramuscular ketamine-HCl (100 mg/ml, 10 mg/kg body weight, Parke Davis &Co.) and intravenous thiopentone sodium (12.5 mg/kg

body weight, May & Baker). Pre-existing ocular pathology was excluded after pupillary dilatation (tropicamide 1%, Alcon) by examination with an operating microscope (Zeiss) and indirect ophthalmoscope (American Optical). Fundus photography (Kowa) was also carried out in each case. Under sterile operating conditions, 0.15 ml aqueous humour was aspirated via the limbus with a 27 gauge disposable needle and 1 ml syringe. The collapsed anterior chamber was reformed with an equal volume of balanced salt solution. Next, 0.4–0.5 ml vitreous humour was aspirated from the centre of the vitreous compartment using a disposable 23 gauge needle connected via a three-way stop-cocked tap to a 1 ml syringe. By altering the tap, it was then possible to replace the aspirated liquid vitreous humour with an equal volume of balanced salt solution or hyaluronic acid (10 mg/ml; Hyvisc®, Pitman Moore, or Healon®, Pharmacia, U.S.A.). In each monkey, samples of aqueous and vitreous humours were obtained from both eyes on three occasions, at day 0, 2 and 14. For comparative purposes human ocular fluids were also obtained from normal eyes undergoing surgery and from cadaver eyes.

Analyses of aqueous and vitreous humours. Samples were clarified by centrifugation at $48\,000 \times g$ for 1 h at 4°C. Carrier-free $Na^{125}I$ (17 Ci/mg iodine) and ^{59}Fe ($FeCl_3$, 54 Ci/g Fe) were purchased from Amersham International (U.K.). Protein was measured colorimetrically by the method of Lowry et al. [7] using bovine serum albumin as standard. Transferrin and lactoferrin were isolated from human and monkey serum and milk as described previously [8]. Radiolabelling of proteins with ^{59}Fe and ^{125}I was performed as described by van Bockxmeer et al. [9,10]. Antisera to purified transferrins and lactoferrins were raised in New Zealand white male rabbits. Immunoelectrophoresis was performed on 1% agarose gel film (Corning, U.S.A.) using 0.2 M barbital buffer, pH 8.6. Sample wells were loaded with 2 μl antigen (1 mg/ml physiological saline) or 10 μl aqueous or vitreous humours followed by electrophoresis for 1 h. Longitudinal wells were charged with 20 μl antiserum. Films were washed, air dried and finally stained with Coomassie brilliant blue R250 [11]. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [12] using 14.5 cm × 26 cm × 0.75 mm slabs containing a linear concentration gradient in acrylamide from 6%*T* to 18%*T*. Molecular weight standards in the range M_r 14300 (lysozyme) to M_r 220000 (myosin) were obtained from Bio-Rad (Richmond, CA, U.S.A.). Gels were run until dye-out under constant voltage (210 V); fixed and stained using either the method of Vesterberg [11] or with the ultra-sensitive silver based method of Merril et al. [13]. The iron-binding capacity of vitreous humour was estimated as described in the results section of this paper. Gel filtration on Sephacryl S-200 (Pharmacia, Sweden) on 0.9 × 26 cm columns was performed according to the manufacturer's recommendations.

Results

Protein composition of monkey vitreous humour

In adult Rhesus monkeys the volume of liquid vitreous humour is 0.8 ml to 1.2 ml [14] of which only 0.4–0.5 ml can be readily withdrawn using a needle and syringe. The soluble protein content of normal vitreous humour was found to be 217 μg/ml ± 4.6% ($n = 20$ eyes, mean ± S.E.). This value is about 1/300 th that of normal plasma, and 2/3rds that of normal cerebrospinal fluid. SDS-polyacrylamide gel electrophoresis, using Coommassie blue R-250 staining, reveals a simple electrophoretic pattern of soluble proteins in normal vitreous humour (Fig. 1). The major proteins identified, on the basis of their molecular weight, are albumin (M_r 68000) and transferrin (M_r 78000). Electrophoretograms obtained using Merrill's [13] ultra-sensitive silver stain reveal a much more complex pattern of proteins in the same sample. In addition to the principal components, albumin and transferrin, two other protein species of M_r 137000 and 16000 as well as trace amounts of proteins in the molecular weight range 107000 to 13000 were detected. The distribution of these components in vitreous humour was estimated by densitometry to be: albumin 40%; presumptive transferrin 30%; M_r 16000 component 15%; M_r 137000 component 10% and the minor components of M_r 107000 to 13000 5–10%. All of these components were identical in electrophoretic mobility under these conditions to those found in monkey serum, although their relative abundance

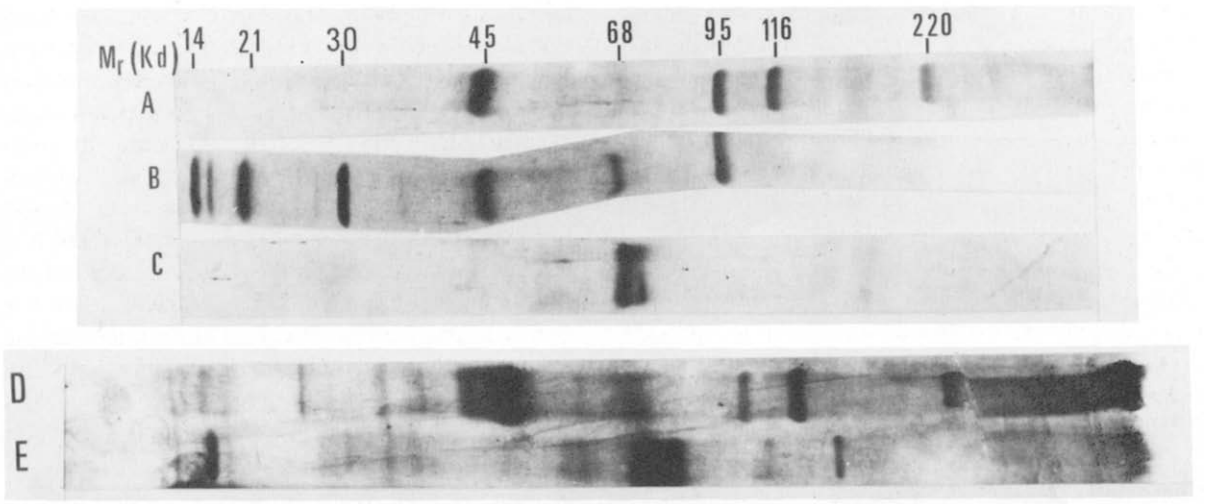

Fig. 1. Protein composition of monkey vitreous humour. SDS-polyacrylamide gel electrophoresis of monkey vitreous humour; lane A, high molecular weight markers; lane B, low molecular weight markers; lane C, 13 μl vitreous humour; lane D, high and low molecular weight markers; lane E, 3μl vitreous humour. Lanes A, B and C were stained with Coomassie brilliant blue R-250 and D and E with a silver-based method [13]. The relative mobility of molecular weight markers (Kd) is noted on lane A.

was markedly different. For example, the ratio of presumptive transferrin: albumin in vitreous humour was 13-times that of monkey serum.

Two days after vitreous humour substitution inflammatory reactions of varying degrees of severity were observed. These cleared in all eyes by 6–7 days with the exception of two from which positive bacterial (*Pseudomonas* and *flavobacterium*) cultures were grown. Analysis of vitreous humour samples showed that the severity of the inflammatory response to vitreous humour replacement paralleled the protein content of vitreous humour and that the transferrin: albumin ratio was decreased relative to normal vitreous humour. In severe inflammation the value for this ratio tended towards that found in serum.

Iron-binding capacity of monkey vitreous humour

^{59}Fe(III)/^{56}Fe(III) nitrilotriacetate was added to vitreous humour samples under conditions known to result in specific iron labelling of unsaturated plasma transferrin [9,10]. The amount of iron added was 30% greater than that required to fully saturate the presumptive vitreal transferrin assuming it to be in the apo form. A 2-fold molar excess of nitrilotriacetate: Fe was also used to prevent nonspecific binding of ^{59}Fe and excess ^{59}Fe to vitreal proteins. Following incubation for 24 h at room temperature the reaction mixture was fractionated on Sephacryl S-200. The elution profile (Fig. 2) consisted of two ^{59}Fe-containing peaks. The first peak co-eluted with ^{125}I-labelled, Fe_2 human transferrin as shown in a separate column run. SDS-polyacrylamide gel electrophoresis analysis showed that it consisted of albumin and the presumptive transferrin. The second peak emerged at the total bed volume of the column and probably consisted of low molecular weight ^{59}Fe-chelate material. Samples containing ^{59}Fe-labelled vitreous protein were concentrated by vacuum dialysis and their ^{59}Fe content measured. These samples were then exhaustively dialysed against buffer solutions containing 50 mM EDTA in the pH range 3.6–8.0. At pH 5.5, a rapid loss of 18% of the ^{59}Fe content was observed. Under identical conditions in the presence of EDTA a sample of purified human transferrin lost most (> 80%) of its iron. To effect near complete removal (> 90%) of ^{59}Fe from the vitreous humour preparation dialysis at pH 3.6 in the presence of EDTA was required. Following dialysis against phosphate-buffered saline at pH 7.4, the samples were re-saturated with ^{59}Fe and fractionated on Sephacryl S-200 as described above. The ^{59}Fe content of the

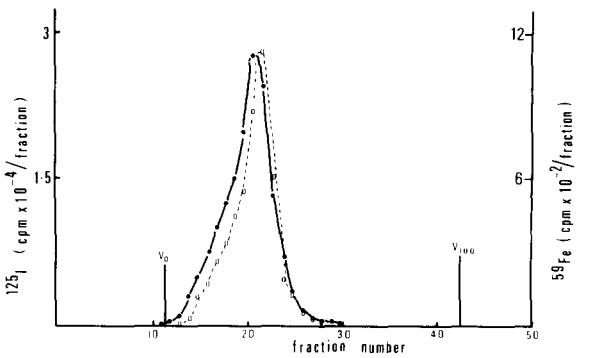

Fig. 2. Iron-binding proteins in monkey vitreous humour. Elution profile of vitreous humour previously incubated with ^{59}Fe on Sephacryl S-200. Protein-bound ^{59}Fe (○·····○) eluted at the same position as ^{125}I-labelled human transferrin (^{125}I-HTN) (●———●). Non-protein bound ^{59}Fe (not shown) emerged near the total bed volume (V_{100}).

resultant protein peak was taken to represent 100% saturated vitreous humour transferrin. Thus, the original vitreous humour sample was conservatively estimated to be only 37% saturated with iron. The true value of the degree of iron saturation of vitreous humour is probably much lower for the following reasons. Vitreous humour samples analysed contained only about 20 μg of presumptive transerrin which would require only 30 ng Fe for full saturation. Reagent contamination with Fe, especially in the last dialysis step prior to ^{59}Fe addition, would decrease the apparent iron-binding capacity of the preparation. Recovery of material from the dialysis sac and following gel filtration would not be completely quantitative resulting in a further underestimate of the iron-binding capacity of the preparation. Given the mean values for monkey vitreous humour volume (1.0 ml), presumptive transferrin content (65 μg/ml), and its estimated latent iron-binding capacity of 63%, the total iron-binding capacity of normal monkey vitreous humour (one eye) is at least 60 ng Fe. This is equivalent to the total haemoglobin iron content of 570000 monkey erythrocytes (mean cell haemoglobin = 21 pg).

Isoelectric focusing and immunoelectrophoresis

The pH dependence of iron-binding by vitreous humour protein suggests the presence of both transferrin and lactoferrin. Lactoferrin may be distinguished from transferrin immunologically and by its ability to bind iron in acid environments. The p*I* lactoferrin is considerably more basic than that of transferrin in all species so far studied [15–18]. Isoelectric focusing in polyacrylamide slab gels (pH 3.5–9.5) of native vitreous humour failed to produce discrete protein bands. Isoelectric focusing of the concentrated first ^{59}Fe-containing Sephacryl S-200 peak showed the presence of albumin (p*I* 4.9) and a series of fainter bands at pH 5.3, 5.7 and 6.9. The proteins of p*I* 5.3 and 5.7 were tentatively identified as apo and iron-transferrin and the p*I* 6.9 potein as lactoferrin. Attempts at isolating pure monkey milk lactoferrin were unsuccessful. The ^{59}Fe-binding protein isolated from milk was shown by immunoelectrophoresis to be exclusively transferrin rather than lactoferrin. Fig. 3 shows the results obtained with immunoelectrophoretic analysis of human and monkey aqueous humour, vitreous humour and subretinal fluid. Single precipitation arcs were observed with purified antigens indicating specificity of antisera. At pH 8.6, the electrophoretic mobility of pure human transferrin was much greater than that of lactoferrin (Fig. 3A). Immunoelectrophoretic analysis of human aqueous humour, vitreous humour and subretinal fluid clearly demonstrated that lactoferrin was a major component. In freshly obtained human vitreous humour, lactoferrin was by far the dominant iron-binding protein present whereas in vitreous humour obtained from cadaver eyes stored for up to 36 h at 4°C, near equal amounts of lactoferrin and transferrin were observed (Fig. 3B). Human aqueous humour samples also showed near equal amounts of lactoferrin and transferrin. The electrophoretic mobility of lactoferrin in human aqueous humour, vitreous humour or subretinal fluid was approximately the same as that of serum transferrin. Similar results have been reported for the electrophoretic behaviour of lactoferrin in other biological fluids [18]. Antisera raised against the human antigens cross-reacted with the monkey proteins. Immunoelectrophoresis using anti-human transferrin and lactoferrin resulted in a faint lactoferrin precipitation arc and two more prominent transferrin arcs (results not shown). Fig. 3C shows that the transferrin in native monkey vitreous humour exhibited an anomolous electro-

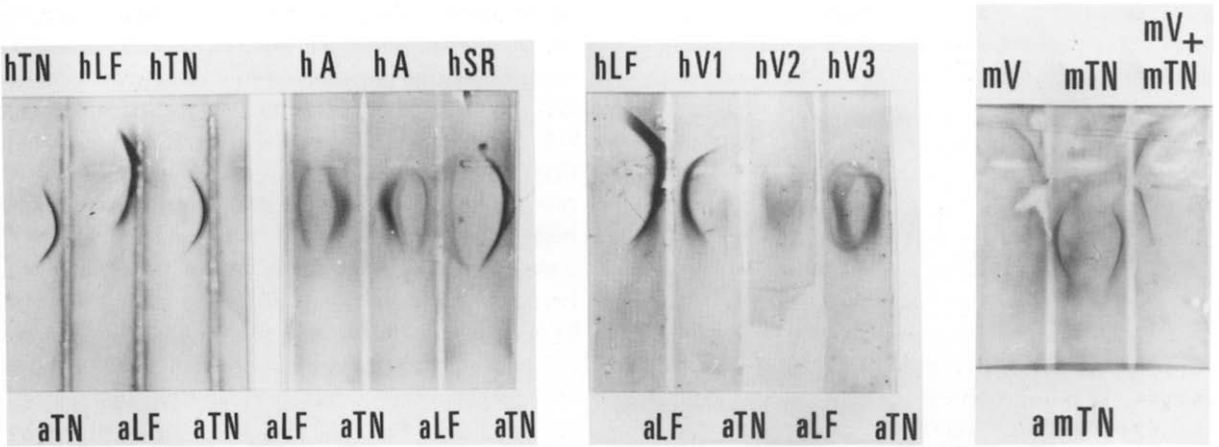

Fig. 3. Immunoelectrophoresis, aqueous and vitreous humours and subretinal fluid. Samples; A (left): human transferrin (hTN), human lactoferrin (hLF), freshly obtained human aqueous humour (hA) and human subretinal fluid (hSR). B (middle): human lactoferrin (hLF), freshly obtained human vitreous humour (hVI) and human cadaveral vitreous humour (hV2, hV3). C (right): monkey vitreous humour (mV) and monkey transferrin (mTN). Antisera used for A and B: anti-human transferrin (aTN) and anti-human lactoferrin (aLF); C: anti-monkey transferrin (amTN).

phoretic behaviour at pH 8.6. That is, transferrin precipitation arcs occurred both cathodally and anodally to the point of protein application. The electrophoretic mobility of purified monkey transferrin, however, was the same as that of human transferrin. Addition of purified monkey transferrin to native monkey vitreous humour prior to immunoelectrophoreis altered the electrophoretogram obtained (Fig. 3C). With increasing amount of transferrin added to vitreous humour, precipitation arcs anodal to the application point increased in intensity until the major arc coincided with that of pure monkey transferrin. The cathodal arcs persisted indicating the presence of an acidic species of transferrin in monkey vitreous humour.

Discussion

The current view on the origin of soluble vitreous humour proteins is that they arise by a process of ultrafiltration from serum through blood ocular barriers [1–4]. This concept is challenged by the discovery of proteins in vitreous humour which do not appear to be present in serum [3,4,6]. One explanation for this finding, which does not seem to have been considered in the literature, is that these so called 'eye-specific proteins' may well be present in serum but at levels which are below the detection limits of the methods used. Their presence in intraocular fluids at high levels (relative to serum) may be due to active transport processes. We find, in agreement with the results of Balazs and co-workers [14,19] that the soluble protein content of monkey vitreous humour is much lower than previously reported for other species [2]. Based on the assumption that these proteins arise solely by passive penetration into vitreous humour from blood, we calculate a blood-vitreous humour filtration quotient of 1/300 rather than 1/50–1/100 as previously reported.

Electrophoretic analysis of monkey vitreous humour showed that 70% of the proteins consisted of near equal amounts of albumin and a protein of identical molecular weight to transferrin. This finding is in agreement with that reported for rabbit vitreous humour where a transferrin-like component represented 26% of the total soluble proteins in vitreous [5]. Ignoring charge differences, but taking into consideration the difference in size between albumin and transferrin and assuming passive penetration of both proteins from blood into vitreous humour, transferrin would be expected to exhibit the same vitreous humour:serum ratio as albumin. However, our re-

sults show that the ratio of presumptive transferrin to albumin in vitreous humour is about 13-times that of monkey serum. That is, for every transferrin molecule there are 20 molecules of albumin in serum and only 1.57 albumin molecules in vitreous humour. Thus, relative to serum, monkey vitreous humour contains a large abundance of presumptive transferrin. These calculations suggest either an intraocular production of transferrin or an active blood-to-vitreous humour transport mechanism to account for the abundance of this protein. Other possibilities include that the vitreous humour component is a protein unique to the eye and of identical molecular weight to transferrin and/or that it consists of lactoferrin as well as serum transferrin.

The iron-binding, isoelectric focusing and immunoelectrophoresis studies reported here indicate that the abundant M_r 78000 component in vitreous humour consists of both serum transferrin and lactoferrin. We have also detected lactoferrin in other intraocular fluids including human aqueous humour, vitreous humour and subretinal fluid. These results indicate that the abundance of the 78 kDa component in vitreous humour is primarily due to the presence of lactoferrin rather than transferrin. While retaining its immunological identity, lactoferrin in intraocular fluids exhibited an anomalous electrophoretic mobility indicating its interaction and binding of acidic moieties present in these fluids. This phenomenon has also been described for lactoferrin in other body fluids and secretions [18,20]. Surprisingly, transferrin in monkey vitreous humour also exhibited anomalous electrophoretic behaviour consistent with an apparent acidification of this protein by vitreous humour. These results also confirm the finding by Chen and Chen [2] that vitreous humour proteins in the dog are acidic, compared with serum proteins, with pI values less than 6.0. In view of the very low lactoferrin content of serum (< 1 μg/ml) [21] and its known sites of synthesis in neutrophilic granulocytes and glandular structures involved in external secretions [22,23] the aqueous humour and vitreal lactoferrin described here is most likely produced within the eye. We suggest the ciliary body as a possible source of ocular lactoferrin as it has a recognised secretory function [24].

Intravitreal haemorrhage is a major clinical problem in diseases such as diabetes mellitus, other retinal vascular disease, penetrating eye injuries and retinal detachment [25–27]. A study involving 100 diabetic patients with vision 20/400 showed that 35% were blinded by intravitreal haemorrhage that failed to clear [28]. This visual impairment has been attributed to iron which may be released during haemoglobin degradation following intravitreal haemorrhage [25–31]. The fate of blood in the vitreous humour is poorly understood and unpredictable. Clinical observations and studies on experimental models indicate that clearance of blood from vitreous humour is a slow process with a time constant of the order of 1% per day [31,32]. Intact red cells have been demonstrated in vitreous humour for at least five months after the haemorrhagic episode [33]. The major mechanism for blood removal appears to be one of erythrophagocytosis [26,31].

The degree of red cell lysis within the vitreous humour and the fate of released haemoglobin are not known. The retention of ^{59}Fe in the retina after clearance of intact ^{59}Fe-labelled red blood cells suggests that some degree of haemolysis occurs [31]. The minimal dose of blood, haemoglobin or iron required to cause retinal toxicity is not known. Limited data from 10 patients requiring vitrectomy for recurrent intravitreal haemorrhage indicate that from 13–542 μl blood was present in the vitreous humour [32]. The average volume of recently haemorrhaged blood in these patients was estimated to be 43 μl. Experimental models for intravitreal haemorrhage generally use from 100–200 μl blood [29–31]. Clearly, post-haemorrhagic iron toxicity would be determined by the mass of red blood cells involved; their clearance rate; the degree of haemolysis and haemoglobin degradation; and, the clearance rates of breakdown products.

The iron-binding studies reported here indicate that vitreous humour lactoferrin-transferrin has a high latent iron-binding capacity of at least 60 ng Fe per monkey eye. This is equivalent to the total haemoglobin iron content of 570000 erythrocytes or 0.1 μl blood. In view of the slow erythrophagocytotic clearance of blood, the persistence of intact red cells and the apparent low degree of haemolysis occurring in vitreous humour, the

amount of haemoglobin iron released is probably a very small fraction of that available at any one time. We suggest that the observed spontaneous clearance of intravitreal haemorrhage without retinal damage is due to the ability of vitreal transferrin-lactoferrin to sequester the otherwise toxic iron released. Should factors or pathological conditions prevail which promote red cell lysis and haemoglobin degradation such that more than 60 ng of free iron is available in vitreous humour, then iron toxicity may occur. Besides this suggested protective function for transferrin and lactoferrin in intravitreal haemorrhage, lactoferrin may also play a protective role in intraocular infections. Lactoferrin is a recognised bacteriostatic agent by virtue of its iron chelating ability under the conditions of bacterial infections [34–36].

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