Ultrastructural evidence of transplacental transport of immunoglobulin G in bitches

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In dogs, passive immunity is conferred to fetuses and neonates by the transfer of maternal immunoglobulin G through the placenta during the last trimester of pregnancy and via the mammary gland after parturition, respectively. However, morphological evidence of transplacental transport is still lacking. The aim of the present study was to localize maternal immunoglobulin G in the labyrinthine zone and in the haemophagous zone of the canine placenta by means of immunohistochemistry and immunocytochemistry. In the labyrinthine zone, immunoglobulin G was detected in all the layers of the materno–fetal barrier including the fetal capillaries. Immunoreactivity was particularly prominent in maternal basement membrane material as well as in the syncytiotrophoblast. However, this evidence of transplacental transport of immunoglobulin G originated from a limited number of unevenly distributed maternal vessels only. In the cytotrophoblast of the haemophagous zone, immunoglobulin G was localized to phagolysosomes at various stages but was never detected within fetal vessels. The results indicate that maternal immunoglobulin G is degraded in cytotrophoblast cells of the hemophagous zone and, therefore, that transplacental transport is restricted to a subpopulation of maternal vessels in the labyrinthine zone.

Materials and Methods

Materials and tissue fixation

Light microscopy was used to detect endogenous IgG originating from the placentas of three bitches of different breeds. These bitches were ovariohysterectomized under...
Fig. 1. Paraffin wax sections of canine placenta. (a) Low-power micrograph showing subdivision of canine placenta into labyrinthine (lb) and haemophagous (hz) zones, myometrium (mm) and glandular chambers (gc) using Goldner staining. Scale bar represents 1 mm. (b) Higher magnification of region enclosed by the box in (a). Arrows indicate peculiar maternal vessels of large diameter near the glandular chambers. Scale bar represents 500 μm. (c, d) Localization of IgG in placental labyrinthine zone after immersion fixation. Indirect immunogold labelling.
Materno-fetal transport of IgG in bitches

inhalation anaesthesia between days 45 and 50 of pregnancy upon request of their owners (mean duration of gestation is 63 days). In two of the bitches, one of the uterine horns was perfused after surgery via the uterine artery. After the tissue was cleared of blood with PBS or Hank’s buffered salt solution (HBSS; Hollweg and Buss, 1980), fixation was achieved by perfusion for 10–20 min with either Bouin’s fixative (Romeis, 1989) or diluted Karnovsky’s fixative (1% (w/v) paraformaldehyde, 1.25% (v/v) glutaraldehyde, 2 mmol CaCl2 l–1 in 0.1 mol cacodylate buffer l–1, pH 7.4), respectively. Fixation was extended by subsequent immersion in the same fixative. Ampullae from the third uterus were cut into strips 1 cm wide running perpendicularly to the girdle zone. These strips were immersed in Stefanini’s fixative, pH 7.4 (Stefanini et al., 1967) for several hours.

Ten millilitres of a 1% (w/v) solution of canine IgG (Sigma, Buchs) in 0.01 mol PBS l–1 was used as a tracer in a 9-year-old beagle bitch presented for euthanasia on day 43 of gestation to ensure a surplus of antibodies in the placenta. After administration of barbiturates, the tracer solution was injected into the uterine artery and, after 5 min, vascular perfusion with diluted Karnovsky’s fixative was performed.

Processing for light microscopy and transmission electron microscopy

Tissue treated with diluted Karnovsky’s fixative was immersed in 0.2 mol glycine l–1 in 0.1 mol cacodylate buffer l–1, pH 7.2 for 30 min before embedding in either paraffin wax or LR White (Polysciences, Eppelheim). Paraffin wax sections (7 µm) of the labyrinthine and hemophagous zones were produced from tissue samples of about 5 mm × 10 mm × 15 mm. Small tissue blocks (approximately 1 mm × 1 mm × 2 mm) from the one placenta that had been perfused with exogenous canine IgG were processed according to standard protocols and embedded in LR White to obtain semithin and ultrathin sections.

Immunohistochemistry

Paraffin wax sections were mounted on glass slides, deparaffinized with xylene and rehydrated through a descending ethanol series. These sections were stored in 0.01 mol PBS l–1, pH 7.2, at 4°C until use. Incubation procedures for immunohistochemistry of paraffin wax and LR White sections are shown (Tables 1 and 2). Fifteen nanometre colloidal gold-conjugated anti-rabbit IgG was purchased from Aurion EM reagents (Wageningen), 3,3’DAB-tetrahydrochloride from Catalys (Wallisellen), and the silver enhancement kit for light and electron microscopy from British Biocell International (Cardiff). All other reagents were obtained from Sigma. After incubation, paraffin wax sections were counterstained with nuclear fast red and LR White sections were stained with toluidine blue.

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<td>Blocking step</td>
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<tr>
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<td>Washing step</td>
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DAB, diaminobenzidine; HRP, horseradish peroxidase; RT, room temperature.

Immunocytochemistry

Ultrathin sections (70 nm) from LR White-embedded material were mounted on copper or nickel single slot or 100 mesh grids. For immunocytochemistry, incubations were the same as for semithin sections (see Table 2). However, a 15 nm gold-conjugated secondary antibody was used instead of the 10 nm gold conjugate. Silver enhancement was used for all

Table 1. Incubation protocol for paraffin wax sections (HRP–DAB labelling)

with silver enhancement on paraffin wax section was used, counterstained with nuclear fast red. Scale bars represent 200 µm. (c) IgG is detected within all maternal vessels of the labyrinthine zone. (d) Negative control on consecutive section to (c). (e) Placental labyrinthine zone after perfusion fixation. Materno-fetal transfer of IgG does not originate from all maternal vessels. Indirect immunogold labelling with silver enhancement on paraffin wax section was used, counterstained with nuclear fast red. Scale bar represents 50 µm.
semithin sections. Gold labelling was also enhanced by silver amplification on some ultrathin section to allow visualization of labelling at low magnification in electron microscopy. Selected ultrathin sections were further vapour osmicated and counterstained with an aqueous solution of uranyl acetate.

Negative controls included the omission of primary antibody and the substitution of the primary rabbit antibody to dog IgG with equivalent concentrations of normal rabbit serum, normal rabbit IgG (not shown) and the use of a rabbit antibody against glial fibrillary acidic protein as an irrelevant surrogate.

Silver staining

For comparison with IgG localization, perivascular basement membrane material was visualized on paraffin wax sections with silver methenamine according to the modified Jones method (Garvey, 1996). Canine kidneys were used as a positive control.

Results

Naturally occurring maternal antibodies had been complemented by exogenous canine IgG in one specimen. Although this resulted in higher detection levels within unperfused maternal blood vessels, the amount and distribution of IgG in maternal and fetal tissues, as measured by immunoreactivity, were identical to that in placentas that contained endogenous IgG only. Therefore, no distinction will be made between the different placentas in the following description of IgG localization.

Labyrinthine zone

After perfusion fixation, most maternal vessels in the labyrinthine zone were empty and thus devoid of IgG. However, in immersion-fixed material, all the vessels were clearly immunoreactive (Fig. 1c,d). Materno–fetal transfer of IgG was evident in the labyrinthine zone of all samples studied but always took place only in a limited number of unevenly distributed vessels (Figs 1e and 2). On the basis of vessel localization and the structure of the vascular wall, no common characteristics emerged. Detection of label in the trophoblast did not depend on blood remaining within the maternal vessels during fixation. On the contrary, IgG was often detected around vessels that had been emptied during perfusion fixation, and evidence of transplacental transport of IgG was no more frequent in the vicinity of non-perfused vessels in which IgG was still available.

Some striking vessels were observed near the glandular chambers. Although these vessels were lined by an endothelium only, their diameter was large and they were surrounded by a thick, compacted trophoblast layer (Figs 1 and 3). When involved in materno–fetal transfer of IgG (which was not always the case), these vessels were outlined by a distinctive circular layer of labelling from which radiating branches extended (Fig. 3a,c). Staining with silver methenamine showed that the presence of basement membrane material was restricted to the immediate vicinity of the vessel (Fig. 3b,d). Therefore, distribution of IgG extended beyond the area occupied by endothelial basement membrane material.

In labyrinthine areas where transfer of IgG occurred, paraffin wax and semithin sections revealed immunolabelling of maternal endothelial cells, underlying basement membranes, syncytiotrophoblast, and fetal capillaries (Figs 4 and 5).

Immunoelectron microscopy studies revealed that IgG was detected in all the layers of the materno–fetal barrier of the labyrinthine zone (Fig. 6). Labelling density was highest in basement membranes (Fig. 6c). With respect to the syncytiotrophoblast, considerable amounts of IgG were confined to vesicles and granules as well as to tubulovesicular profiles near the fetal endothelium (Fig. 6c–e). In addition, some gold particles that could not be

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*Ultrathin sections.

Optional.

EM, electron microscopy; LM, light microscopy; RT, room temperature.
related to any organelles were localized within the cytoplasm (data not shown).

With respect to maternal endothelial cells, most of the label was scattered within the cytoplasm (Fig. 6b). In addition, there were some immunoreactive membrane-bound granules (Fig. 6b, inset). Intercellular spaces were devoid of label. A low labelling intensity was found in fetal endothelial cells and capillary luminae (Figs 5 and 6).

The control sections incubated in the absence of primary antibodies were negative (Figs 4b, 5b, 7e and 8b). Controls using normal rabbit serum (Fig. 7b) or normal rabbit IgG (data not shown) instead of rabbit anti-dog IgG were also negative but for a well-delineated labelling of specific cell organelles, for instance the nuclei of epithelial cells in the glandular chambers. Thus, no staining of any structures visualized by the specific primary antibody was seen using normal rabbit serum. Use of rabbit antibody against glial fibrillary acidic protein instead of the specific primary antibody did not produce any signal (Fig. 7d). Furthermore, immunolabelling of the maternal blood was entirely restricted to the blood plasma between the erythrocytes (Fig. 7f).

**Haemophagous zone**

Since the marginal haematoma cannot be flushed by perfusion fixation, tissue preservation of the haemophagous zone was not as good as it was in the labyrinthine zone.

Immunolabelling of the marginal haematoma itself was very clearly limited to the blood plasma (Fig. 8). Within the cytotrophoblast cells of the haemophagous zone, IgG was located exclusively to granules of variable size arranged into columns (Fig. 8). Small granules of homogeneous optical density were labelled evenly in paraffin wax and semi thin sections (Fig. 8a). In large granules, a patchy labelling seemed to outline discrete spherical contents.

Evidence of phagocytosis of erythrocytes by cytotrophoblast cells was provided by electron microscopy (Fig. 9). Phagosomes and phagolysosomes containing erythrocytes in various stages of degradation were found in almost every cell. In these organelles, IgG was sparse and was found mainly in the spaces between phagocytosed red blood cells.

Erythrocytes in the early stages of degradation showed no immunolabelling, while some immunolabelling was

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**Fig. 2.** Localization of IgG in canine placenta by indirect immunogold labelling with silver enhancement on paraffin wax sections, counterstained with nuclear fast red. The vessels from which materno–fetal transfer of IgG originates are constant both in the two consecutive sections (a) and (b) and on a section (c) taken 400 μm apart from (b). Scale bars represent 200 μm.
associated with later stages (Fig. 10). Electron microscopy confirmed high labelling density in multivesicular bodies and in small granules of homogeneous density (Figs 11).

No evidence of IgG was found in fetal endothelia and capillaries of the haemophagous zone. Control reactions in the haemophagous zone were also negative (Fig. 8b).

**Discussion**

Besides postnatal transfer of maternal IgG through the colostrum, some antigen-specific passive immunity is conferred to dog fetuses during the last third of pregnancy (Schneider and Szathmáry, 1939; Gillespie et al., 1958; Krakowka et al., 1978). However, no morphological evidence has been provided yet on the location or mechanism of materno–fetal transport of IgG.

The present study demonstrates IgG transport through all the layers of the materno–fetal barrier in the labyrinthine zone. However, IgG was detectable only in the vicinity of a limited number of maternal vessels. In all samples studied, these vessels were unevenly distributed in the labyrinthine zone. A fixation artifact may account for this unexpected distribution pattern. However, labelling was identical with all fixatives used. Assuming a general fixation problem, leakage of IgG through the vascular wall should be expected to occur in poorly fixed areas. However, evidence of materno–fetal transfer of IgG was no more frequent in tissue fixed by immersion than it was in perfusion-fixed material. Furthermore, label was regularly found in well-perfused maternal vessels in which IgG was no longer available after the onset of fixation. Even in immersion-fixed tissue in which blood obviously remained available within the vessels, the presence of IgG outside maternal vessels was no more common than it was in perfusion-fixed placentas. Moreover, label was associated with well-defined organelles reflecting active uptake. Taken together, these results provide strong evidence against a fixation bias. Umbilical cord vessels reach
the girdle placenta at its antimesometrial side (Starck, 1959), while maternal stem vessels approach it from the mesometrial side. How these vessels branch within the labyrinthine zone is largely unknown but, if areas for IgG transfer are dependent on these topographical relationships, the stray distribution pattern may be the result of a hidden design being masked by unconsidered sampling.

Static images can provide no more than a fragmentary view of dynamic events such as the materno–fetal transport of IgG. However, the consistent occurrence of typical labelling intensities for the different layers of the materno–fetal barrier is likely to reflect variations in transport pace through the various compartments. Immuno-

labelling of maternal endothelial cells was moderate but the absence of IgG from all intercellular spaces indicated transcellular transfer. IgG concentration was highest in maternal basement membranes. This finding was not surprising since basement membranes, such as of the glomerular basement membrane, tend to be considerable permeability barriers for proteins (Cotran and Rennke, 1983). In fact, the human trophoblast basement membrane may play a similar role as a filter in the human placenta (Edwards et al., 1993). Antibody titres are comparatively low in newborn puppies (Young et al., 1949; Ott, 1956; Gillespie et al., 1958). Therefore, a low labelling density within fetal capillaries was to be expected. Labelling of the endothelium

Fig. 4. Semithin section showing IgG-positive tissue in the vicinity of a maternal vessel in the canine placental labyrinthine zone. Labelling of semithin sections was used to select areas of interest for electron microscopy as shown in Figs 6 and 8–11. (a) Indirect immunogold labelling with silver enhancement, counterstained with toluidine blue. (b) Negative control on consecutive section. Scale bars represent 50 µm.

Fig. 5. Semithin section of canine placental labyrinthine zone showing maternal vessels (mv) involved in transplacental transfer of IgG. Endothelial cells (arrowheads) and syncytiotrophoblast cells are immunopositive. (a) Epipolarization after indirect immunogold labelling with silver enhancement. (b) Bright field illumination of negative control counterstained with toluidine blue. Scale bars represent 20 µm.
Fig. 6. Ultrastructural localization of IgG in the different layers of the canine materno–fetal barrier of the labyrinthine zone. (b–e) Indirect immunogold labelling followed by vapour osmication and (a) silver enhancement, vapour osmication and staining with uranyl acetate. On favourable sections, the presence of IgG was detected throughout all the layers of the placental barrier from maternal to fetal blood (a). In maternal endothelial cells, IgG was usually scattered within the cytoplasm (b) but occasionally was found in electron-dense granules (inset); note the absence of label over the nucleus in (b). Labelling density was particularly high in basement membrane material (c, arrowheads). Within the syncytiotrophoblast, IgG was confined to vesicles and granules (d) as well as to tubulovesicular profiles extending up to the fetal endothelium (e). fv, fetal vessel; me, maternal endothelium; st, syncytiotrophoblast. Scale bars represent (a,d) 5 μm, (b,e) 1 μm and (c) 2 μm.
Fig. 7. Control reactions demonstrating specificity of immunolabelling in canine placental labyrinthine zone. (a–e) Sections from identical regions of the placental labyrinthine zone were taken between the sections shown in Fig. 2a and c and were counterstained with nuclear fast red. (a) Indirect immunogold labelling with silver enhancement; (b) normal rabbit serum used instead of primary antibody; (c) silver enhancement alone; (d) a rabbit antibody against glial fibrillary acidic protein used as an irrelevant primary antibody; and (e) omission of primary antibody. Scale bars represent 100 μm. (f) Contents of maternal vessel in the labyrinthine zone revealing high specificity of IgG labelling. Indirect immunogold labelling with silver enhancement was used, an ultrathin section was vapour osmicated and stained with uranyl acetate. Scale bar represents 2 μm.

Fig. 8. Semithin sections of the haemophagous zone of the canine placenta. Within the marginal haematoma, immunoreactivity is restricted to the space between red blood cells (arrows). Whereas labelling is homogenous in smaller granules, IgG distribution is patchy in large phagolysosomes containing erythrocytes (arrowheads point to corresponding organelles). (a) Indirect immunogold labelling with silver enhancement, counterstained with toluidine blue. (b) Negative control on consecutive section. fv, fetal vessels; mh, marginal haematoma. Scale bars represent 20 μm.
itself was sparse. However, the cytoplasm of fetal endothelial cells was extremely scant, indicating that selective transcellular transport might again occur very rapidly. In humans, human immunoglobulin 2 (hIgG2) reaches the fetal capillary lumen in far lower amounts than does hIgG1 as a result of the selective properties of the fetal capillary endothelia (Mongan and Ockleford, 1996). Since IgG did not accumulate around canine fetal capillaries, exclusion of specific IgG subclasses does not seem to occur in dogs.

In humans, transplacental transfer of IgG is the main mechanism by which passive immunity is conferred to the progeny and requires the transport of substantial amounts of protein (Kohler and Farr, 1966; Pitcher-Wilmott et al., 1980; Malek et al., 1996). However, despite extensive work, including the identification of a plethora of Fcy receptors (Kameda et al., 1991; Bright et al., 1994; Saji et al., 1994; Story et al., 1994; Simister, 1998), the mechanism of transplacental transport remains far from clear (Wild, 1981; Malek et al., 1997; Simister and Story, 1997; Hunziker and Kraehenbuhl, 1998; Landor et al., 1998). The mechanism by which IgG is taken up by the syncytiotrophoblast and transported from the stroma to the lumen of fetal blood vessels is also unclear. Current working models assume that, after reaching endosomes either by fluid-phase (Leach et al., 1991) or receptor-mediated endocytosis (Johnson and Brown, 1981; King, 1982; Sibley and Boyd, 1992; Landor et al., 1998; Malek et al., 1997), IgG is rescued from the default pathway to lysosomes by the neonatal Fc receptor (FcRn) and is then delivered to the interstitial fluid of the stroma (Lin, 1980; Simister and Story, 1997). In the present study, IgG was found in vesicles likely to be endosomes and in tubulovesicular profiles next to the fetal endothelium. This finding is in agreement with the current concept of transplacental transfer. However, the IgG concentration in canine umbilical cord blood amounts to no more than 2–18% of the maternal IgG concentration (Schneider and Szathmáry, 1939; Gillespie et al., 1958; Krakowka et al., 1978), which explains the comparatively low abundance of label found within the canine placenta.

Transport of IgG from maternal blood to the extracellular spaces of the trophoblast was also frequently observed around the large vessels in the basal parts of the labyrinthine zone. Unfortunately, these vessels could not be identified before embedding and so no semithin or ultrathin sections could be obtained. Thus, precise localization of IgG remains tentative. However, comparison of immunolabelled and silver methenamine-stained consecutive paraffin wax sections revealed that IgG did extend beyond the endothelial basement membrane material and into what are likely to be clefts between units of the syncytiotrophoblast. Analogous findings have been reported in the haemochorial placentas of different species in which a paracellular transport route has been suggested in addition to transcellular transport (Stulc, 1989). The fibrin-containing deposits at discontinuities in the syncytiotrophoblast may be the morphological equivalents of a paracellular transport route in the human placenta.
In dogs, fetal capillaries are virtually absent from the thick layer of trophoblast surrounding these peculiar maternal vessels. Therefore, these areas are unlikely to make a substantial contribution to the net transfer of IgG from mother to young.

Cytotrophoblast cells of the haemophagous zone of the dog placenta phagocytose maternal erythrocytes (Burton, 1982) to supply the fetus with iron. Plasma containing IgG is present in the phagosomes with the red blood cells, as reflected by the preferential immunolabelling of the spaces between phagocytosed erythrocytes. As breakdown progresses, the contents of the phagolysosomes are concentrated and intermingled so that later degradation stages of red blood cells become increasingly associated with gold label. The smaller granules of homogeneous electron density observed probably represent phagosomes lacking a red blood cell, which is consistent with their uniform labelling for IgG. The fate of these phagosomes is likely to be the same as for the phagolysosomes. Since no evidence of IgG was found in any other organelle or beyond the cytotrophoblast cells of the haemophagous zone, immunoglobulins are probably degraded within the phagolysosomes, with all the other contents.

In summary, this study provides ultrastructural evidence that materno–fetal transfer of IgG during the last third of pregnancy in dogs originates from a subpopulation of maternal blood vessels in the labyrinthine zone only. No indication of transplacental transport was detected in other regions of the canine placenta. Further studies are needed to elucidate the mechanism of transport and its restriction to individual vessels.

The outstanding technical assistance of C. Furer and of C. Hug and the excellent photographic work of S. König are gratefully acknowledged.

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**Fig. 11.** Cytotrophoblast cells of haemophagous zone of the canine placenta. Note the absence of labelling in the cytoplasm (a) and the even distribution of IgG in phagosomes that do not contain any erythrocytes (a,b). Scale bars represent (a) 1 µm and (b) 0.5 µm.
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