THE RECENT DISCOVERY OF WHAT MIGHT BE A PRIMORDIAL ESCAPE MECHANISM FOR *TRYPANOSOMA CRUZI*

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"Where did the parasites learn their immunology? Not from Roit, not even the latest edition; nor from Immunology Today; and not even from attending useful symposia. Rather, they have learned their craft in the tough classroom of Natural Selection."

R. J. Terry

Deane, Lenzi & Jansen (1984) have recently described a double cycle of *Trypanosoma cruzi* in one of its most common natural hosts, the opossum *Didelphis marsupialis*: the vertebrate cycle involving a systemic infection with blood invasion by trypomastigotes and an invertebrate cycle, occurring in the lumen of the anal glands, with abundant multiplication in the epimastigote stage and transformation into a metacyclic stage. Organisms of both cycles were found to be infective to vertebrate (mice) and invertebrate (triatomines) hosts (Thomaz et al., 1984).

The implications of this finding are manifold as it opens to investigation new chapters related to the general biology of *T. cruzi*, the immunology of the infection and the epidemiology of Chagas' disease (Deane et al., 1984, this Symposium; Lenzi, Jansen & Deane, 1984). One of the most fascinating aspects is the survival of the parasite in stages that are normally completely defenseless against both unespecific and specific (immune) factors present in blood and tissues of the vertebrates, a fact that suggests a high degree of adaptation of the parasite to this host.

The relationship between *T. cruzi* and marsupials is probably quite ancient. Fossil evidence indicates that these mammals originated in the New World, possibly in what is now South America, in the Cretaceous period (Kirsh, 1977). More than 70 species are now found in the American Continent, most classified in the Family Didelphidae, which is thought to be the oldest living group (Kirsh, 1977). In the 12 genera ascribed to the Family, several species have been found naturally infected with *T. cruzi*, frequently in high proportions (Barretto, 1979; Barretto & Ribeiro, 1979). This is particularly true of the genus *Didelphis* that includes three generally recognized species, *D. virginiana*, *D. marsupialis* and *D. albiventris*. The first record of *T. cruzi* in *D. marsupialis* was by Robertson, in Honduras (1929). From then on the association *T. cruzi-Didelphis* sp. (all three species) has been reported by a large number of workers from North, Central and South America (Barretto, 1979) and rates of infection have reached more than 90% in some places (Alencar et al., 1981).

To the omnivorous diet of didelphids is partially attributed their long survival in evolutionary terms. This diet includes fruits, grains, eggs, small vertebrates and invertebrates. Arthropods, specially insects, have been found to make up for more than 50% (dry weight) of the food remains in opossum stomachs or scats and, among the insects, the orders Hemiptera (including the "assassin bugs", or Reduviidae) and Diptera were included (Reynolds, 1945; Sandidge, 1953). It should be reminded that it is among these two orders that monogenetic Trypanosomatidae are most frequent (Wallace, 1966).

In captivity opossums promptly attack and devour living mice and triatomine bugs and can acquire *T. cruzi* in both ways (Yeager, 1971; Carrera et al., 1983).

It has been speculated that the tissue protozoa started their parasitic life by the route of the alimentary tract of the vertebrate host, with or without the intervention of insects. For Trypanosomatidae, insectivorous feeding habits of vertebrates could have offered the opportunity for adaptation of monogenetic stocks to a digenetic cycle with the secondary adaptation to blood-sucking insect vectors. At this moment, we do not wish to dwell in such speculations which are discussed by Baker (1963), Wallace (1966), Hoare (1972), Lainson & Shaw (1979), among others. However, one is tempted to suggest that a location such as the anal glands — which are like intestinal appendages and in direct contact with the intestinal lumen — could have afforded a convenient stepping-stone for the passage from a strickly intestinal habitat to the blood and tissues of the opossum. It is generally recognized that intraluminal location in hollow vertebrate viscera, offers a relative protection to the parasites against the "full consequences of immune sensitization" (Cohen, 1982), of which a monogenetic protozoon coming from an insect intestinal tract would have no "memory", or previous experience.

All the opossums found with heavily infected anal glands had circulating anti-*T. cruzi* antibodies and all had been able to control their (experimental) infection to sub-patent levels, with long periods of negative parasitological examinations (Deane, Lenzi & Jansen, 1984; Deane et al., 1984).

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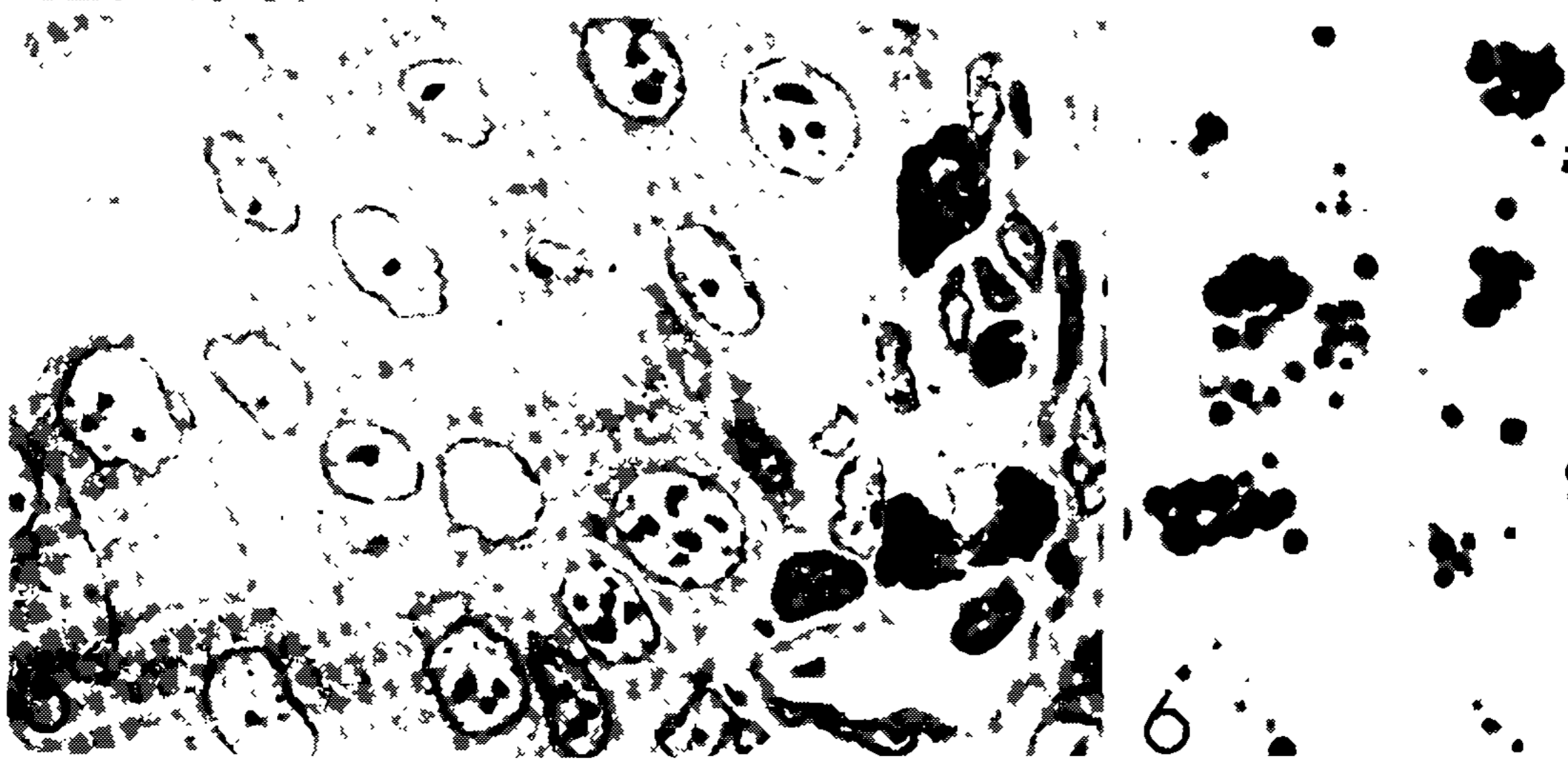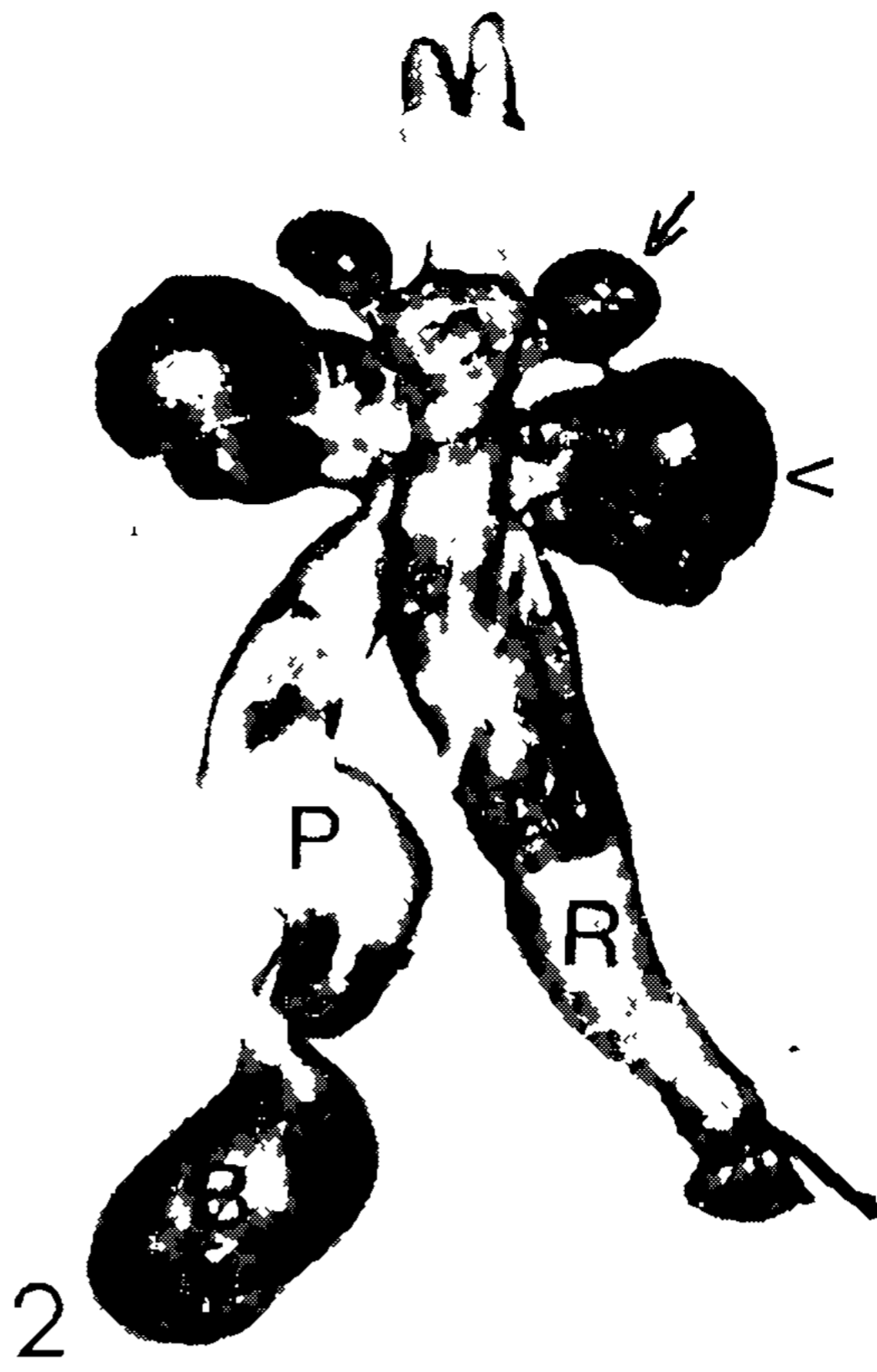
Fig. 1: anal (odoriferous) glands (arrow) in schiopubic region with their ducts (arrow-head) draining to the anus. The glans penis is bifid. Fig. 2: anal glands in the males close to the three pairs of bulbourethral glands. B: urinary bladder. P: prostate gland with three distint zones. R: rectum. Fig. 3: mucosa, submucosa and thick layer of striated muscle composing the wall of the anal glands. The superficial layer scales off epithelial cells with signs of degeneration. HE X 200. Fig. 4: stratified epithelium rich in papillary projections with an exuberant capillary network. Fovea or pit is the space confined by the papillary projections. Gomori's reticulum strain. X 200. Fig. 5: epon-embedded semi-thin section of stratified epithelium showing the dark basal cells and the clearer and voluminous superficial cells. In the connective axis of the submucosa, there are four mastocytes around vessels. Methylene blue − azure II− basic fuchsin stain. X 1250. Fig. 6: epon-embedded semi-thin section showing lipid inclusions of different sizes (dark droplets) in the epithelial cells. Snipes' stain. X 1250.

In the vertebrate tissues, *T. cruzi* disposes of various evasion mechanisms, such as intracellular location; shedding and renewal of surface antigens; antibody cleavage; immunosuppression; modification of leucocyte function and polyclonal lymphocyte activation (Vianna, 1911; Silverstein, Steinman & Cohn, 1977; Nogueira & Cohn, 1976; Leon et al., 1979; Schmunis et al., 1980; Krettli, Thomaz & Eisen, 1980; Ramos et al., 1978, 1979; Rowland & Kuhn, 1978; Cunningham & Kuhn, 1980; Nogueira, Chaplan & Cohn, 1980; D'Imperio Lima et al., 1984). However, none of these mechanisms is known to be effective for the protection of extracellular epimastigotes. Furthermore, as already stressed (Deane, Lenzi & Jansen, 1984), in this phase the trypanosome is rapidly lysed by complement or engulfed and destroyed by phagocytes of non-immune hosts. Opossum complement is as efficient as human or guinea-pig's in lysing culture epimastigotes of *T. cruzi* (Thomaz & Deane, unpublished).

Which is (or are) the factor (or factors) that protects *T. cruzi* epimastigotes in the lumen of the opossum's anal glands? Could this be a primordial evasion mechanism and the "missing-link" that could help to understand adaptation from a monogenetic invertebrate cycle to a digenetic parasitism?

To answer these and other questions several investigations are under way, including a a detailed study of the anatomy, histology and histochemistry of the glands, the physical relationships of the parasites with the glandular structure and possible ways by which the parasites penetrate and survive inside the glandular lumen. A brief account on some of these aspects is here presented.

The glands

Both male and female opossums present a pair of odoriferous glands which are called "anal" or "ad-anal". Figs. 1-7 show the anatomical relationships of these glands and aspects of their histology. The proximity of the glandular ducts to the anal orifice (Figs. 1 and 2), and the presence of a rich capillary bed (Fig. 4) are stressed. The glandular epithelium exhibits apocrine and holocrine secretions and scales off superficial cells rich in lipids; the remains of these cells together with large quantities of lipid droplets and an alcian-blue pH 2,5 staining substance are found in the glands lumen (Figs. 5 and 6).

Intraluminal parasites – physical relationships with glandular structures

The fact that six out of eight *D. marsupialis*, infected with "G-N" and "G-49" strains, lodged the parasites in the lumen of their anal glands indicates a repetitive event and not a randon occurrence. While a detailed and systematic search in a great number of sections of other organs or tissues of these opossums was negative, in paraffin sections of their anal glands abundant flagellates were found. The parasites were not seen in the central region of the gland but close to the surface of the epithelium that covers the pits or foveas (Figs. 8 and 9), free in the lumen (Figs 10 and 14), or attached to integral or deformed cells, or in direct contact with large droplets cast of the glandular epithelium (Figs. 10, 11 and 13). In paraffin sections the parasites looked like amastigotes (Figs. 8 and 9), but in semithin and ultrathin sections they varied from small, round to large elongate epimastigotes and, rarely, trypomastigotes (Figs. 10-14). As already mentioned elsewhere (Deane, Lenzi & Jansen, 1984) in fresh preparations of some infected glands, amastigotes were not found and trypomastigotes of the metacyclic type were very numerous.

Importance of the anal glands

Factors such as abundance of required nutrients, trypanosoma attractans, tissue receptors, the vascular component of the glands and local temperature could be involved in this peculiar localization of *T. cruzi*. Temperature might be an important factor in the parasite differentiation, the average body temperature for opossums being well below that of entherian mammals (Hunsaker, 1977). For this reason there is little chance to find *T. cruzi* in the human peri-anal or intramuscular glands, which present some characteristics similar to that of the opossum anal glands (Netter, 1962).

The presence of glycosaminoglycans (or proteoglycans) which covers the surface of superficial epithelial cells, detected by alcian-blue pH 2,5 staining, may also in some way be involved in providing a facilitation environment for parasite division and, due to their dense negative charge, may restrict the approach of the parasite to the epithelial cells. Although the intraluminal parasites differentiate to metacyclic forms there is no intracellular invasion or location in scalled or fixed epithelial cells (Figs. 8-10, 12 and 13). The fact that these metacyclic trypomastigotes are infective (Deane, Lenzi & Jansen, 1984) suggests that the glands function as a big reservoir or a "natural culture flask" interposed between the bloodstream and the external environment. This could be very important from the epidemiological point of view, because the parasites presumably can move toward the feces through the glandular ducts after spontaneous contraction of the striated muscular layer. Indeed, when the anal glands of infected opossums were mechanically pressed, parasites were released off through the anus.

The capacity of the anal glands to discharge their infected contents to the exterior could explain the limited epidemic outbreaks of human Chagas' disease occurring in non endemic areas for which oral transmission and direct or indirect participation of opossums were suggested (Nery-Guimarães et al., 1968; Shaw, Lainson & Frahia, 1969; Miles et al., 1978), and the high levels of natural infection among marsupials observed in areas with sparse triatomine distribution (Olsen et al., 1964; McKeever, Gorman & Norman, 1968).
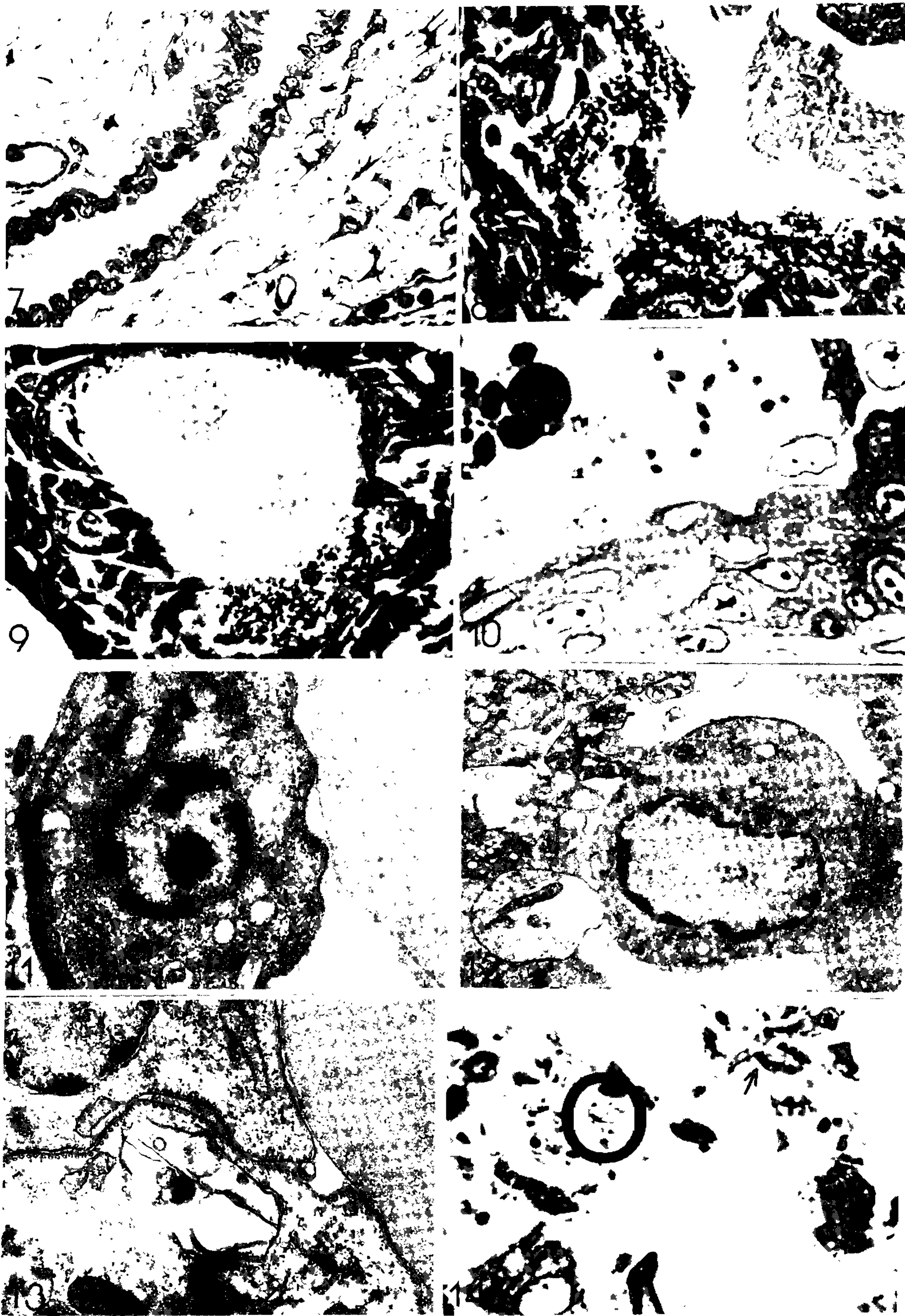
Fig. 7: glandular duct surrounded by connective tissue. Epon-embedded semi-thin section stained by Methylene blue-azure II — basic fuchsin. X 500. Fig. 8 and 9: great number of parasites covering the superficial epithelial cells inside foveas or pits. Toluidine blue. X 500. Fig. 10: epon-embedded semi-thin section illustrating from parasites in the lumen of the anal glands. X 1250. *Inset.* Three parasites stuck on the surface of a secreted globule. X 2000. Fig. 11: detail from the inset of Fig. 10 showing two attachment points on the surface of the secreted globule. The globule is limited by a membrane coated by rests of cytoplasm. Electron microscopy. X 33800. Fig. 12: vacuolated epimastigotes in contact with a superficial cell shedding from the epithelium. Electron microscopy. X 6500. Fig. 13: two parasites in opposition on the surface of a globule of secretion. The subpellicular microtubules of the parasites are well visualized. Electron microscopy. X 33800. Fig. 14: vacuolated epimastigotes (arrow) and one trypomastigote (circle) observed in epon-embedded semi-thin section. Methylene blue — azure II — basic fuchsin stain. X 1250.
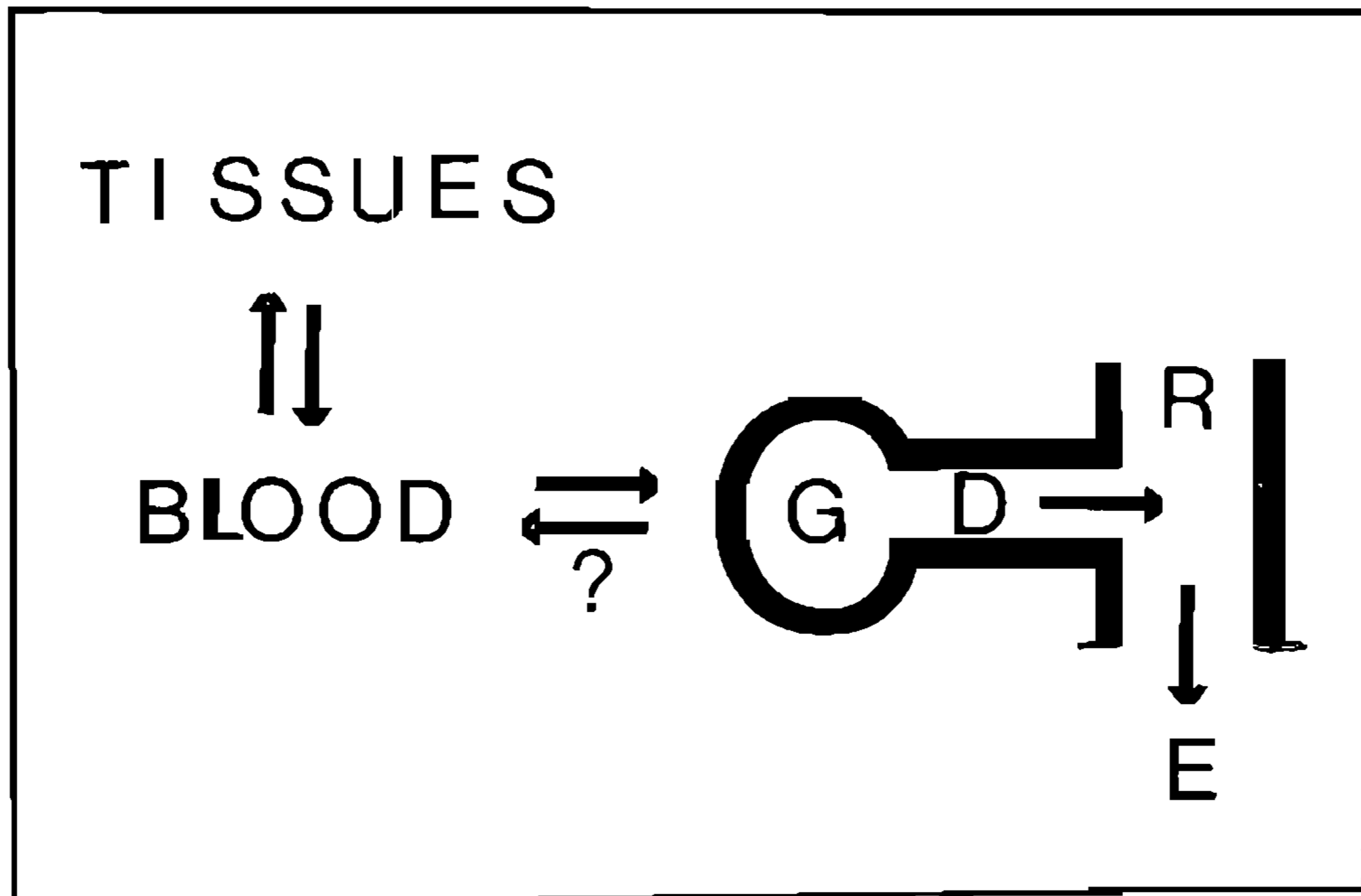
Fig. 15: drawing showing the anal gland (G) interposed between the blood-stream and external environment (E) through its duct (D). Parasites presu-mably, can circulate back and forth from the bloodstream to the tissues, and from the bloodstream to the anal gland. The duct allows the parasite to be expelled to the exterior (E). R: rectum.

On the other hand, the abundant capillary network of the glands provides an easy way by which the parasites may come from the bloodstream toward the lumen of the gland and, probably, can move back into the bloodstream. This could explain the apparent disappearance of the parasites from the peripheral blood, to appear again later. Mastocytes, frequently observed close to vessels of the submucosa and in the axial tissue papillary projections (Fig. 5), could have a participation in this event, through their several vascular mediators. By way of the capillary network, antigens produced inside the glands during parasitic proliferation and differentiation may also be periodically or continuously released into the bloodstream, causing immunoresponse or, perhaps, tolerance or immunosuppression.

Inside the glands the parasites not only escape from the humoral response as already pointed out, but are remarkably free of cellular attack. There is no mononuclear cell infiltration in the histological layers of the glands, in spite of the cellular competence of the opossums (Block, 1960; La Plante, Taylor & Burrell, 1968; La Plante et al., 1969; Bryant, 1977).

As a final comment, we wish to suggest that the discovery of T. cruzi in the lumen of the anal glands of the opossum offers new approaches for the investigation of immunological interactions between trypanosomatids and their vertebrate hosts.

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