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Permeability alteration of the sarcolemmal membrane, particularly at the site of macrophage contact, in experimental chronic *Trypanosoma cruzi* myocarditis in mice

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Summary. Sarcolemmal membrane permeability characteristics have been investigated, particularly at the site of macrophage contact, in experimental chronic *Trypanosoma cruzi* myocarditis in BALB/c mice, employing ruthenium red (RR) as an electron tracer. The ultrastructural features of the myocardium from infected animals were similar to those previously described. Briefly, focal myocarditis was detected, with areas of myocytolytic necrosis, atrophic myofibres, an inflammatory response composed of mononuclear cells, predominantly macrophages and a few lymphocytes, and interstitial fibrosis. This study provided the following new information: (1) the cytoplasmic components of mononuclear cells have a very high affinity for RR. It is conceivable that mononuclear cell activation parallels a physiological change in plasma membrane permeability; (2) RR diffusely stains the sarcoplasm of cardiocytes with anomalous contraction bands, indicating leaky sarcolemmal membranes; (3) most non-degenerating cardiocytes from experimental animals appear darker with RR staining than controls. They also frequently show rows of RR-stained sub-plasmalemmal tiny vesicles. Both changes probably reflect increased membrane permeability; (4) RR intensely labels the cytoplasmic components of cardiocytes at the site of macrophage contact or close apposition, indicating areas of altered membrane with remarkably increased permeability. This observation provides insight into a role for the macrophages in myocardial cell damage in experimental chronic *Trypanosoma cruzi* myocarditis. An obvious consequence of increased membrane permeability is that it may cause impairment of the transmembrane ion gradients and cause loss of intracellular elements, thus contributing to cardiocyte death. Furthermore, the findings in the present study imply that one of the consequences of macrophage-mediated cytotoxicity may be alteration in the permeability of the plasma membranes of nearby target cells, possibly due to a change in the structural integrity of the membrane resulting from peroxidation of cell membrane lipids.

Keywords: *Trypanosoma cruzi*, chronic myocarditis, macrophage cytotoxicity, cardiac sarcolemma permeability, Chagas' heart disease, ruthenium red

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Immunological mechanisms have been implicated in the pathogenesis of chronic chagasic cardiopathy. The participation of autoimmune mechanisms relies on the myocarditis characterized by an inflammatory infiltrate composed predominantly of mononuclear cells, particularly macrophages and a few lymphocytes, both in man and in laboratory animals (Andrade 1983; Ribeiro-dos-Santos & Rossi 1985). Furthermore, a good correlation has been noted between the foci of myocardial damage, characterized by myocytolytic necrosis and degeneration, and the number of mononuclear cells (Molina & Kierszenbaum 1988). The mechanism of tissue injury may be dependent on sensitization to parasite antigens (Ribeiro-dos-Santos *et al.* 1981) or may be a response to the constituents of *Trypanosoma cruzi* antigenically cross-reactive with myocardial tissue (Teixeira *et al.* 1975). This may be mediated through the immunocompetent cells capable of inducing the lesion. Although the inflammatory response is T lymphocyte-dependent, macrophages appear to be the effector cells (Ribeiro-dos-Santos 1984). Evidence indicates that there are two ways by which activated macrophages may kill target cells: one is by inducing inhibition of DNA synthesis; the other, by releasing lytic substances (Adams 1982; Russell 1986). Since maintenance of normal physiological function by the myocardial cell depends on the integrity of its semipermeable sarcolemma, and increased permeability of the cell membrane to protein molecules, enzymes, and cations is a well established phenomenon accompanying various types of cell injury (Singal & Dhalla 1984), it is clearly important if changes in sarcolemmal permeability accompany chronic chagasic myocarditis.

The present study illustrates both the morphology of the myocardial sarcolemma and permeability changes at the site of macrophage contact in experimental chronic *Trypanosoma cruzi* myocarditis in mice, using ruthenium red as an electron tracer.

Materials and methods

Female BALB/c mice, aged 4–5 weeks, were inoculated with 1000 trypomastigote forms of the Y strain of *Trypanosoma cruzi*. Control mice were injected with saline.

The animals were housed (five or six per cage) in polypropylene cages, maintained under controlled conditions, and given laboratory chow and water *ad libitum*. Forty to 60 days after inoculation the mice were killed under light ether anaesthesia by exsanguination from the abdominal aorta. The thoracic cavity was opened, exposing the still-beating heart. The hearts of nine mice from the infected group and six mice from the controls were taken, and samples of the base and apex of the heart were rapidly excised and processed for electron microscopy.

Small pieces of tissue were fixed by immersion in phosphate-buffered 2.5% glutaraldehyde, pH 7.3, for 2–3 h, postfixed in 1% osmium tetroxide in phosphate buffer for 2 h, dehydrated in graded ethanol and embedded in Epon. Semithin (0.5 μm) sections stained with toluidine blue were examined under the light microscope and a suitable area selected for preparation of ultrathin sections. These were double stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 109 electron microscope at 80 kV.

To visualize permeability alteration of the cardiac sarcolemma, small pieces of the myocardium were immersed into a 1.2% glutaraldehyde–0.3% ruthenium red mixture in 0.1 M cacodylate buffer, pH 7.3 for 1 h at room temperature, rinsed in the buffer, postfixed in a 2% osmium tetroxide–0·3% ruthenium red mixture in 0·1 M cacodylate buffer for 2 h at room temperature, dehydrated through ethanol, and embedded in Epon (Luft 1971a). Unstained 1.5 μm thick sections were prepared for light microscopy. Unstained ultrathin sections, selected on the basis of light microscopy, were examined in the electron microscope. Since this technique

is subject to artefacts because ruthenium red is introduced during the process of immersion fixation with glutaraldehyde, random sampling and blind evaluation were performed in order to control for these effects.

Results

The *T. cruzi*-infected mice showed a mortality rate of 60%. There were no deaths in the uninfected control group. The control mice had no ultrastructural myocardial lesions. Figures 1 and 2 illustrate the appearance of a strong ruthenium red (RR) effect in control mouse myocardium. None of the cardiocytes has been penetrated by the tracer and the density is confined to the extracellular space, particularly the pericellular material and collagen bundles. The T-tubules, a derivative of the plasma membrane that penetrates the myofibre centripetally, showed a strong RR reaction. Occasionally, the sacs of the sarcoplasmic reticulum were also stained. In Fig. 2 a strong RR-positive density is seen around an unstained interstitial cell. Since mechanical damage during trimming prior to fixation causes intracellular labelling in most peripheral cells, the outer 30 μm or so of the tissue block was not considered during the study (Luft 1971b).

The ultrastructural features of the myocardium from infected animals were similar to those previously described (Tafuri *et al.* 1973; Palacios-Prü *et al.* 1982; Rossi *et al.* 1984; Figueiredo *et al.* 1986; Pereira-Barreto *et al.* 1986). Briefly, the myocardial changes varied from one area to another, and from apparently normal to a complete

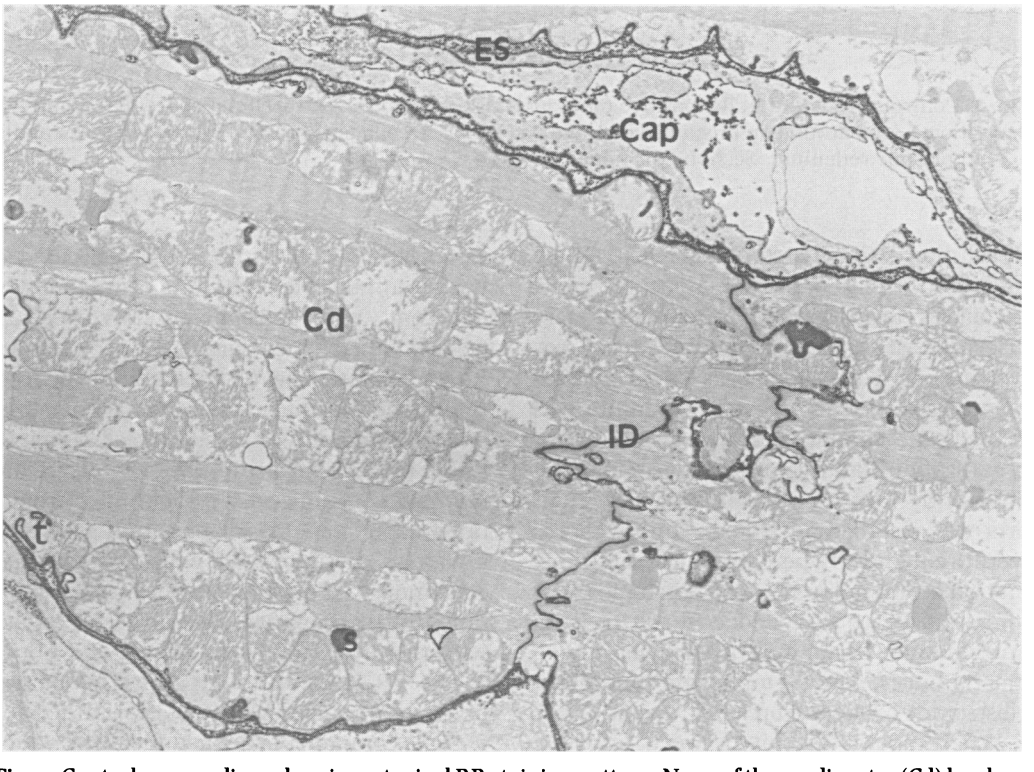

Fig. 1. Control myocardium showing a typical RR staining pattern. None of the cardiocytes (Cd) has been penetrated by the tracer, and the dense stain is confined to the extracellular space (ES). Cap, capillary vessel; ID, intercalated disc; t, T-tubule; s, sarcoplasmic reticulum. Section unstained. × 7100.

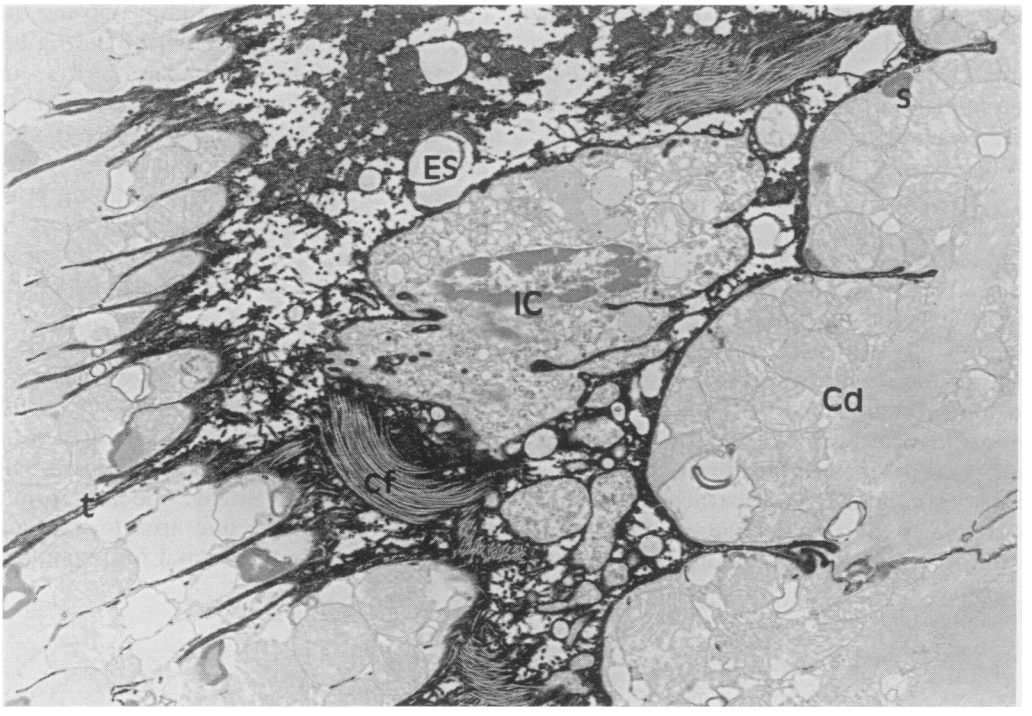

Fig. 2. Control myocardium. RR-positive material is restricted to the extracellular space (ES). Dense RR deposition is seen around an unstained interstitial cell (IC). cf, collagen fibres; Cd, cardiocyte; t, T-tubule; s, sarcoplasmic reticulum. Sections unstained. × 9500.

dissolution of the myocardial fibres. In mildly and moderately damaged areas, atrophic myofibres could be seen. The myofibrils were disorganized, with foci of lysis, there was oedema between myofibrils and myofilaments, and the sarcoplasmic reticulum was dilated. Mitochondria showed swelling, disorganization, and rupture of cristae. An apparently increased number of mitochondria was also demonstrated. The myofibres were frequently hypercontracted, with formation of anomalous contraction bands. The interstitial space was widened due to oedema and massive cellular infiltration consisting of macrophages, a few lymphocytes and fibroblasts with collagen fibres.

When the relationship between activated macrophages and cardiocytes was examined by electron microscopy, a very close proximity between the two cells could be seen. The sarcolemma of the myocardial cells had a wavy outline. The basal lamina was loosely adherent, with a relatively low electron density and a finely granular appearance. Rows of tiny vesicles (caveolae) could be seen under the plasma membrane. The macrophages commonly showed increased convolution of their surfaces in the vicinity of the myofibres. Numerous cytoplasmic projections come into close apposition with the myocytes, many of them with multiple vesicles under the plasma membrane. At other times the macrophages were in close contact with the myocardial cells (Figs 3 and 4).

The RR staining in experimental mouse myocardium demonstrated that the cytoplasmic components of the interstitial mononuclear cells were deeply stained by the tracer. Most of the non-degenerating cardiocytes appeared darker than those from con-

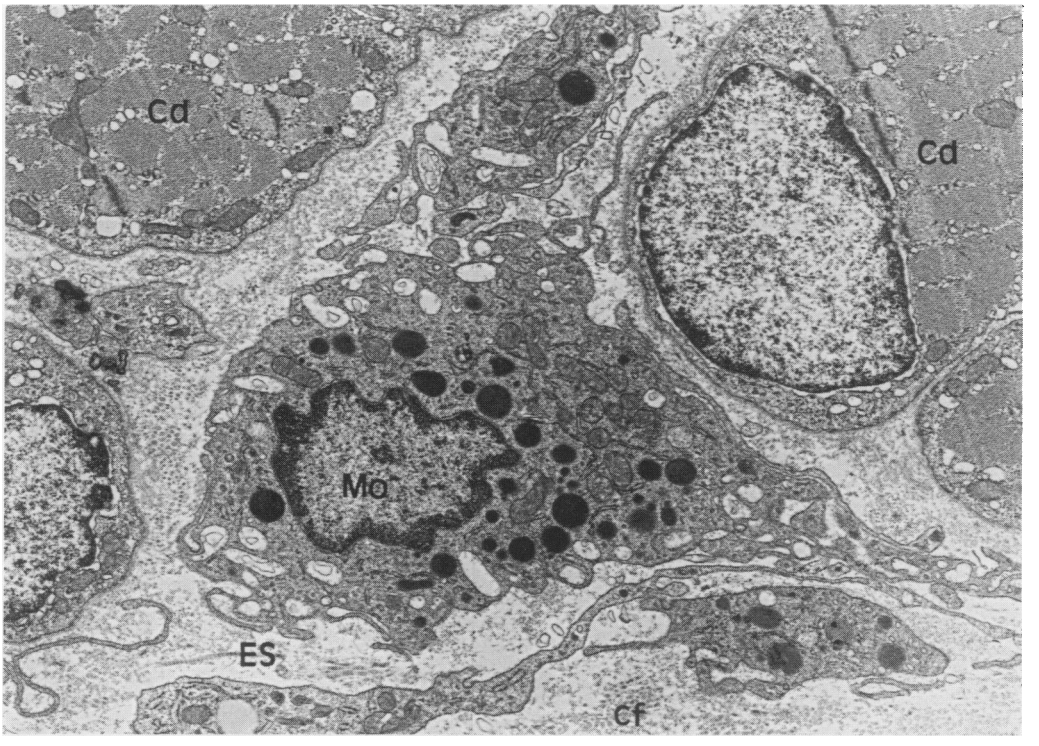

Fig. 3. Myocardium from infected mouse. Macrophages (Mo) show increased convolution of their surfaces in the vicinity of myofibres. Numerous cytoplasmic projections are closely apposed to the cardiocytes (Cd). ES, extracellular space; cf, collagen fibres. Uranyl acetate and lead citrate. ×9500.

trol myocardium and frequently showed rows of RR-stained caveolae (Fig. 5). Myofibres with anomalous contraction bands were permeated by the tracer which strongly stained the hypercontracted myofilaments and mitochondria (Fig. 6).

The appearance of a strong RR effect in experimental mouse myocardium is illustrated in Figs 7 and 8. The cytoplasmic components of the macrophages in close contact with the cardiocytes have taken up RR as shown by their density. In Fig. 7, the myofibre at the bottom of the figure shows intense RR staining of myofibrils, mitochondria and T-tubules, predominantly in the subsarcolemmal zone adjacent to the adherent macrophage. Compare its density, for example, with that of the myocyte in the upper right corner. This myofibre shows a row of RR-stained small vesicles under the plasma membrane. In Fig. 8, a macrophage is in close contact with a deeply RR-stained cardiocyte showing hypercontracted myofibrils. A graded difference in labelling of the cytoplasmic components of this myofibre by the tracer, from the contact zone of the mononuclear cell, can be clearly seen.

Discussion

The ultrastructural changes in the myocardium of the *T. cruzi*-infected mice were similar to those found in previous electron microscopic studies of chronic chagasic cardiopathy both in human patients (Tafuri *et al.* 1973; Palacios-Prū *et al.* 1982; Pereira-Barreto *et al.* 1986) and experimental

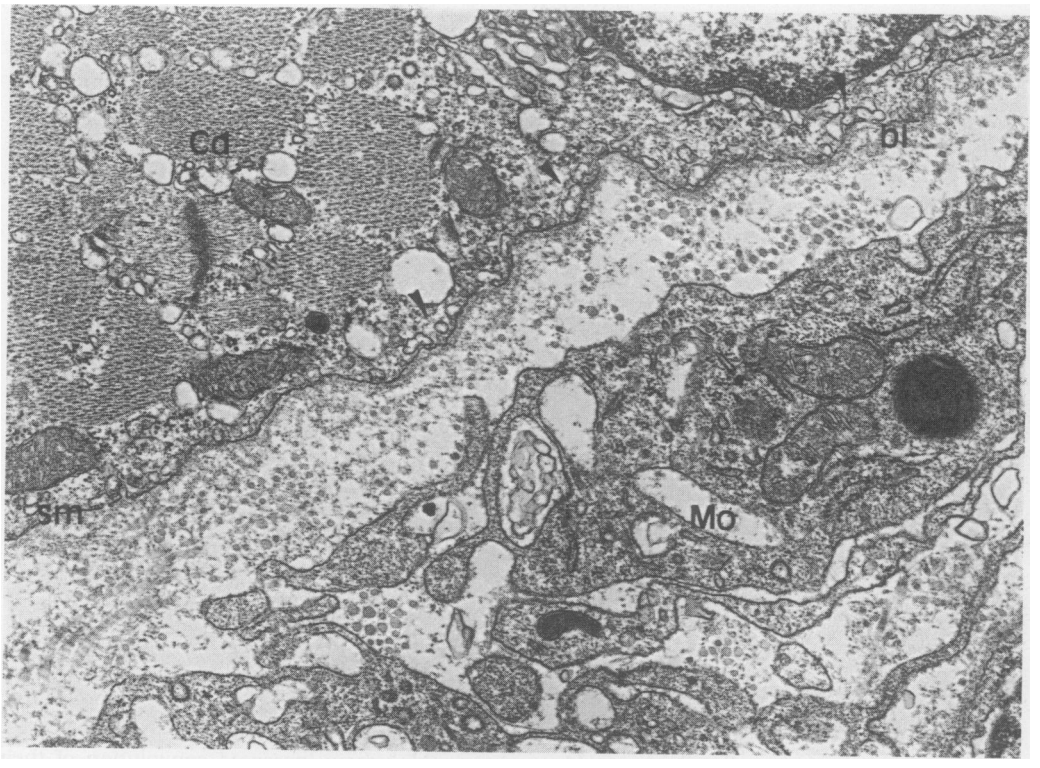

Fig. 4. Myocardium from infected mouse. Cytoplasmic projections of a macrophage (Mo) are in close contact with a myocardial cell (Cd). The sarcolemma (sm) of the cardiocyte has a wavy outline. The basal lamina (bl) is loosely adherent, with a relatively low electron density and a finely granular appearance. Rows of tiny vesicles can be seen under the plasma membrane (arrow heads). Uranyl acetate and lead citrate. × 27 500.

animals (Rossi *et al.* 1984; Figueiredo *et al.* 1986).

In this study, the sarcoplasm of cardiocytes with characteristic contraction band necrosis was consistently stained by the extracellular diffusion tracer, ruthenium red (RR), indicating leaky sarcolemmal membranes (Tani Ametani 1970). These myocytes with RR reaction were located individually or in groups. The mononuclear cells, especially macrophages and a few lymphocytes, were the most frequent component cells of the inflammatory infiltrate in the chronic myocarditis of experimental mice. These cells showed a very high affinity for RR. It is generally accepted that cell cytoplasm does not stain with this tracer if the plasma membrane is undamaged (Tani & Ametani 1970, Luft 1971b). However, the permeability of certain cells is known to vary with functional change (Luft 1971b). It is thus conceivable that mononuclear cell activation in experimental chronic *T. cruzi* myocarditis parallels a physiological change in their membrane permeability. This suggestion is reinforced by the fact that the interstitial myocardial cells in control mice did not show cytoplasmic labelling by RR. On the other hand, areas of altered membrane with increased permeability is the most likely explanation for the darker appearance of most non-degenerating cardiocytes with RR staining in infected animals by comparison with controls. Again, the subsarcolemmal

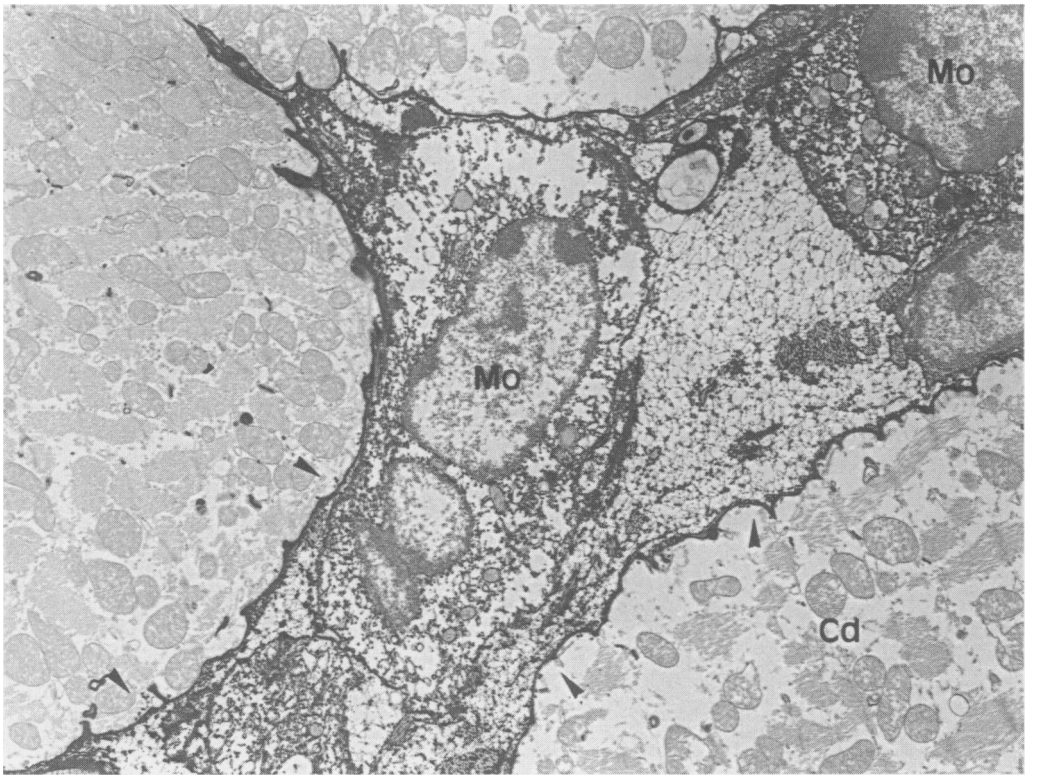

Fig. 5. Myocardium from infected mouse. The cytoplasmic components of the interstitial mononuclear cells (Mo) are deeply stained by RR. The non-degenerating cardiocytes (Cd) appear darker than those from control myocardium. Rows of RR-stained subplasmalemmal small vesicles can be seen (arrow heads). Section unstained. × 7100.

RR-stained vesicles reflect increased membrane permeability.

The present study revealed that macrophages develop increased convolution of their surfaces in the vicinity of the cardiocytes in the myocardium of *T. cruzi*-infected mice and that a number of small vesicles form adjacent to the myofibre. In addition, changes in the permeability of sarcolemmal membranes at the site of macrophage contact or close apposition could be demonstrated. The intense RR labelling of myofibrils, mitochondria, T-tubules, and sarcoplasmic sacs, predominantly in the subplasmalemmal zone adjacent to the adherent macrophage, denotes alteration of the plasma membrane permeability. These observations support the currently adopted hypothetical model of macrophage-mediated cytotoxicity (Evans 1975; Seljelid 1975; Lohmann-Matthes & Fischer 1975; Adams *et al.* 1982; Russell 1986). It is known that direct contact between cytotoxic macrophage and target cell is necessary for killing to occur. During this interaction an increasing number of surface projections develop. Following contact, macrophages secrete lytic effector substances into both the extracellular compartment and, particularly, into the space between macrophage and target. Serum inhibitors neutralize most of the lytic activity in the extracellular compartment, but not in the contact zone, thus permitting the lytic factors to produce target injury. It

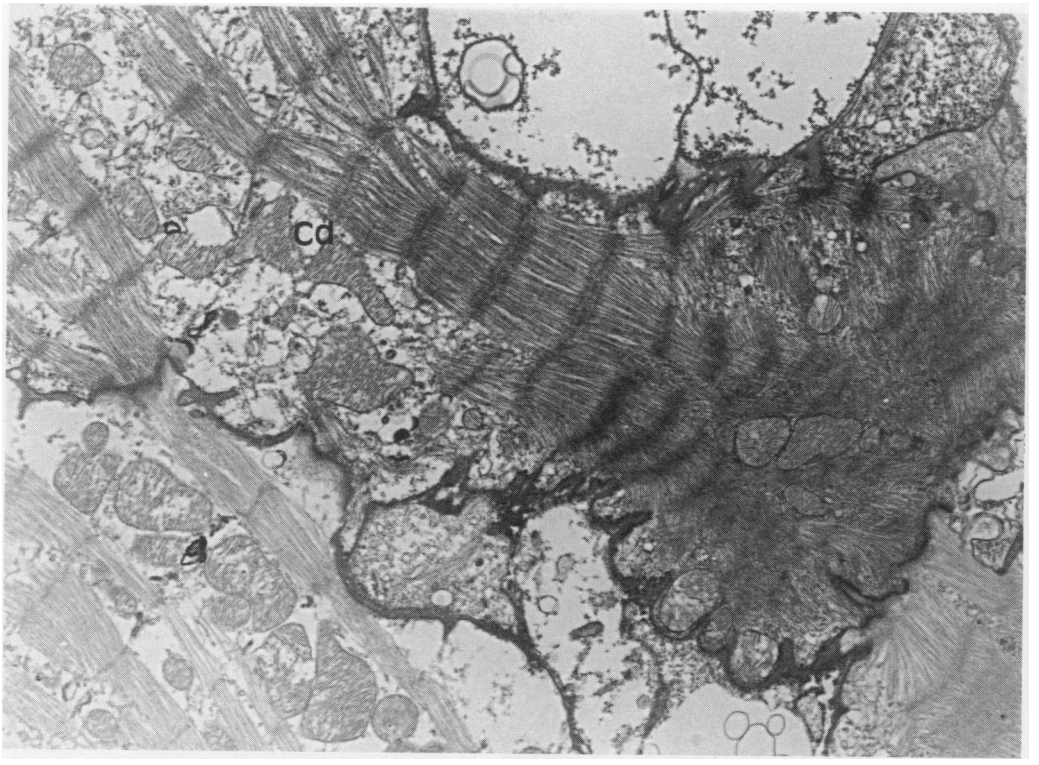

Fig. 6. Myocardium from infected mouse. The cardiocyte (Cd) with anomalous contraction bands is penetrated by the tracer, which has deeply stained hypercontracted myofilaments and mitochondria. Section unstained. ×9500.

should be pointed out that macrophages secrete multiple toxic substances (Nathan 1987), and there is abundant evidence that products of activated macrophages are implicated in many types of tissue damage (Johnson *et al.* 1988). The increased sarcolemmal membrane permeability observed in the chronic myocarditis of *T. cruzi*-infected mice could be a consequence of peroxidative breakdown of membrane unsaturated fatty acids due to the action of oxygen radicals. Macrophages are known to secrete large amounts of oxygen free radicals, and their products, such as interleukin-1 (IL-1) or tumour necrosis factor (TNF), may enhance the generation of oxygen-derived products by macrophages (Ward *et al.* 1988). One of the mechanisms by which oxidants are known to damage cells and tissues is by peroxidization of cell membrane lipids, thus altering the structural integrity of the membrane (Kako 1987).

In summary, this study provides the following new information: (1) the cytoplasmic components of mononuclear cells (macrophages and a few lymphocytes) which are the most frequent inflammatory cell type occurring in the chronic myocarditis of *T. cruzi*-infected mice, have a very high affinity for RR. It is probable that mononuclear cell activation parallels a physiological change in plasma membrane permeability; (2) RR diffusely stains the sarcoplasm of cardiocytes with anomalous contraction bands, indicating leaky sarcolemmal membranes; (3) most non-degenerating cardiocytes from experimental animals stain more strongly with RR than do controls. They also frequently show

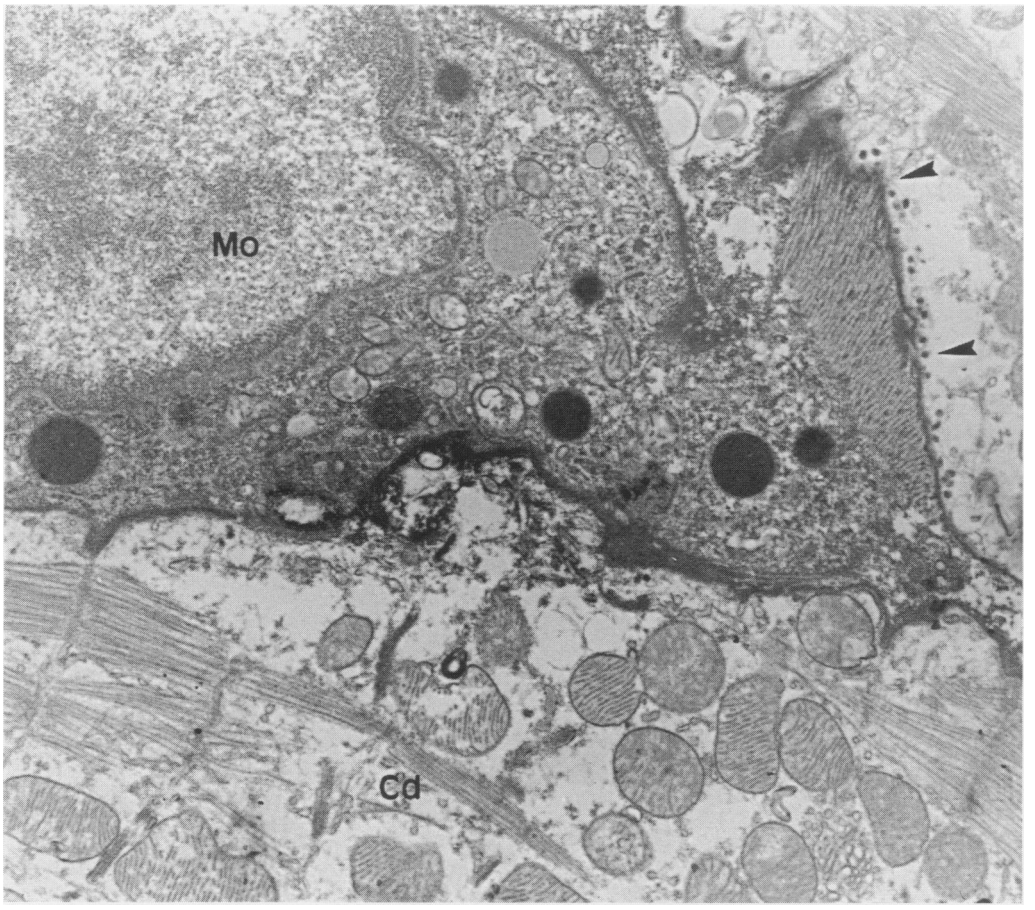

Fig. 7. Myocardium from infected mouse. The cytoplasmic components of the macrophage (Mo) in close contact with cardiocytes (Cd) have taken up RR as evidenced by their density. The myofibre in the bottom of the figure shows intense RR staining of myofibrils, mitochondria, and T-tubules, predominantly in the sarcolemmal zone adjacent to the adherent macrophage. Compare its density with the myocyte in the upper right corner. This myofibre shows a row of RR-stained subplasmalemmal small vesicles (arrowheads). Section unstained. × 24 000.

rows of RR-stained sub-plasmalemmal tiny vesicles. Both changes probably reflect increased membrane permeability; (4) RR intensely labels the cytoplasmic components of cardiocytes at the site of macrophage contact or close apposition, indicating areas of altered membrane with remarkably increased permeability. This observation provides insight into the role of macrophages in myocardial cell damage in experimental chronic *Trypanosoma cruzi* myocarditis. An obvious consequence of increased membrane permeability is that it may impair transmembrane ion gradients and result in loss of intracellular elements, thus contributing to cardiocyte death (Ganote 1983; Frame *et al.* 1983). Furthermore, the present findings imply that one of the consequences of macrophage-mediated cytotoxicity may be alteration of the permeability of the plasma membranes of nearby target cells, due to alteration in the structural integrity of the membrane after peroxidation of cell membrane lipids.

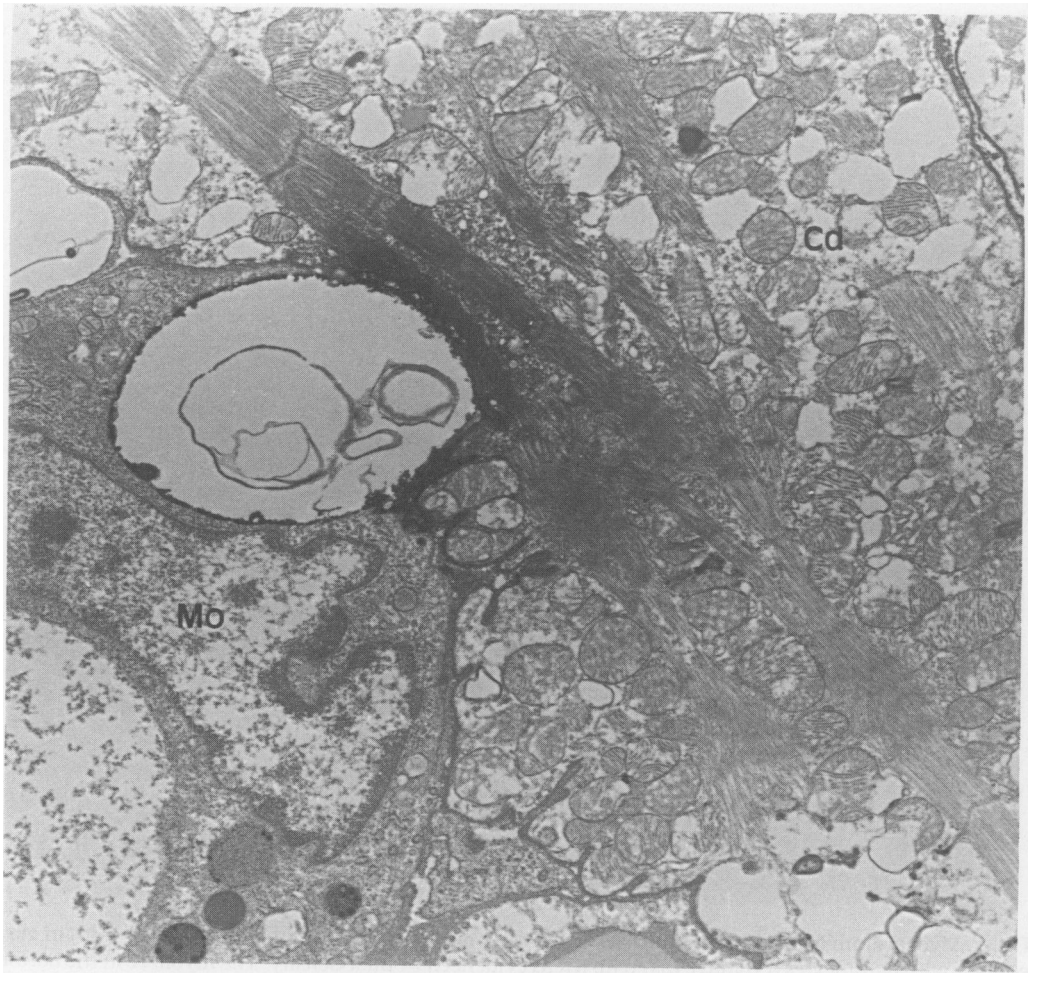

Fig. 8. Myocardium from infected mouse. A macrophage (Mo) is in close contact with a RR deeply stained cardiocyte (Cd) showing hypercontracted myofibrils. A graded difference in labelling of the cytoplasmic components of the myofibre by the tracer, from the contact zone of the mononuclear cell, can be clearly seen. Section unstained. ×9500.

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