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A monoclonal antibody (ER-HR3) against murine macrophages. II. Biochemical and functional aspects of the ER-HR3 antigen

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Abstract. We describe the purification and intracellular distribution of an antigen present on a subpopulation of murine macrophages and recognized by monoclonal antibody ER-HR3 against bone marrow-derived haemopoietic reticulum cells. Using the ER-HR3 antibody as an immobilizing ligand, two proteins were isolated as determined by SDS polyacrylamide gel electrophoresis. Under non-reducing conditions, there was a major band with an apparent molecular mass of 69 kDa and a minor band of 55 kDa. Under reducing conditions, the apparent molecular mass of each band was estimated as 76 kDa and 67 kDa, respectively. Intracellularly, these proteins occurred in close association with membranous structures, as demonstrated with gold-labelled protein A in an electron-microscopic study of the ER-HR3-positive cell line AP284. Some of the antigen was present in vesicles. To gain further insight into the possible function of the ER-HR3 antigen, its tissue distribution was investigated under distinct experimental conditions. In mice infected with Bacillus Calmette Gurèrin, ER-HR3-positive cells were observed in many, but not all, granulomata of the spleen, the lung and the liver. The ER-HR3 reactivity in these mice clearly differed from that of other anti-macrophage monoclonal antibodies, such as F4/80, M5/114 and M1/70. Furthermore, phenylhydrazine-induced extramedullary erythropoiesis in the liver was accompanied by ER-HR3 expression on a subpopulation of macrophages. Finally, the addition of ER-HR3 to an antigen-specific T cell proliferation assay did not inhibit T cell proliferation.

Key words: Macrophages – Monoclonal antibody – Antigen – Purification – Differentiation – Activation – Mouse [(CBA/Rij × C57BL/Rij) F1(BCBA)]

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Introduction

Macrophages are a heterogeneous cell population that is distributed throughout the body (Dougherty and McBride 1984). They express a variety of surface antigens, such as receptors and immunoregulatory molecules (Wright and Silverstein 1986; Gordon et al. 1988). The development of the hybridoma technique has made it possible to produce monoclonal antibodies (mAbs) against these antigens. These mAbs can be used to determine, for example the tissue distribution and maturation state of subclasses of macrophages. However, these histological approaches often do not elucidate the function of a certain antigen and more sophisticated methods are needed.

We have previously described the distribution of a new mAb (ER-HR3) against a subpopulation of macrophages in the fetal and adult mouse (de Jong et al. 1993). Here, we present the results of a number of experiments carried out in order to obtain more information about the function of the antigen recognized by this mAb. We have purified the ER-HR3 antigen, determined its molecular mass and investigated its intracellular distribution. Moreover, we have assessed its expression under experimental conditions in which macrophages might adapt their antigen expression in correlation with altered functions. Such adaptive changes may occur after injection with phenylhydrazine (PHZ) or sublethal doses of pathogenic organisms, such as Bacillus Calmette Guèrin (BCG). Additionally, we have investigated the antigen expression on a number of macrophage cell lines and on macrophages in bone-marrow cultures in order to substantiate the possible relationship between ER-HR3 antigen expression and macrophage differentiation.

Materials and methods

Mice

Male and female (CBA/Rij × C57BL/Rij) F1 (BCBA) mice were obtained from the Radiobiological Institute TNO (Rijswijk, The Netherlands) and kept under conventional conditions.

Table 1. Cell lines and references

Cell line	Cell type	Reference
M1	Myeloblast	Ichikawa (1969)
RMB-1	Myelocyte	De Both et al. (1981)
RMB-3	Myelocyte	De Both et al. (1981)
WEHI-3B	Immature macrophage	Warner et al. (1969)
WEHI-3	Immature macrophage	Warner et al. (1969)
Pu5-1.8	Macrophage	Ralph et al. (1974)
J774-1.6	Macrophage	Ralph et al. (1975)
P388D1	Macrophage	Koren et al. (1975)
RAW264.7	Macrophage	Raschke et al. (1978)
RAW309Cr.1	Macrophage	Raschke et al. (1978)
WR19M.1	Macrophage	Raschke et al. (1978)
AP284	Macrophage	Klasen et al. (1988)

Cell lines

The classification and original references of the macrophage cell lines used are depicted in Table 1. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. The cell line AP284-D4 was isolated in our laboratory from the spleen of a BCBA mouse.

Immobilization of ER-HR3 on protein A-Sepharose

The ER-HR3 antibody was purified and immobilized as described by de Jong et al. (1993) and Schneider et al. (1982), respectively. Briefly, purified antibody was mixed with protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) in 0.1 M borate buffer, pH 8.2, and incubated for 30 min at room temperature under gentle shaking. Beads were successively washed with borate buffer and 0.2 M triethanolamine, pH 8.2. They were subsequently resuspended in 20 ml 50 mM dimethyl pimelimidate dihydrochloride (Pierce, Rockford, Ill., USA) freshly made up in 0.2 M triethanolamine with the pH re-adjusted to 8.2. The mixture was agitated gently at room temperature for 1 h. The reaction was stopped by centrifuging the beads (500 × *g* for 1 min) and resuspending them in an equal volume of 50 mM ethanolamine, pH 8.2. After 5 min, the crosslinked beads were washed three times with borate buffer, pH 8.2, supplemented with 0.02% sodium azide.

Isolation and characterization of the ER-HR3 antigen

Cell suspensions of the spleen and terminal ileum were obtained by cutting these organs into pieces and pushing them through a mesh sieve. After centrifugation (200 × *g*, 15 min, 4°C),the cell pellet was dissolved in a lysis buffer of 1% (v/v) Triton X-100, 1 mM phenylmethylsulphonyl fluoride [PMSF, stock: 2 mg PMSF/ml dimethyl sulphoxide (DMSO)], 1 mg/ml leupeptin (Sigma, St. Louis, Mo., USA) and 1 µg/ml pepstatin (Sigma) in 0.1 M phosphate-buffered saline (PBS). The cellular fragments were further homogenized in a Dounce homogenizer by 20–30 fast strokes with a tight-fitting pestle. The homogenate was centrifuged (1500 × *g*, 30 min, 4°C) and the supernatant was diluted by addition of an equal volume of 0.1 M phosphate buffer, pH 8.0. Cellular proteins binding to protein A-Sepharose were removed by pre-clearance of the solution on a protein A-Sepharose column. The eluate was subsequently layered onto an ER-HR3-Sepharose column. Bound proteins were removed with a 0.1 M glycine/HCl buffer, pH 2.5. The eluate was neutralized with 0.1 M TRIS/HCl buffer (pH 8.5), concentrated in an Amicon filtration unit and processed for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The separated proteins were electrophoretically transferred to nitrocellulose (Towbin et al. 1979) and immunologically detected as described below.

Spot-test

Aliquots of 1 µl of the affinity-purified cellular proteins and control solutions were spotted directly onto nitrocellulose sheets. Sheets were successively incubated for 15 min in PBS, pH 7.8, supplemented with 0.02% (w/v) gelatin and 0.05% (v/v) Tween-20 (PBT), for 1 h with ER-HR3, rinsed with PBT, and finally incubated for 1 h with a 1:40 diluted solution of a peroxidase-conjugated rabbit-anti-rat antibody (Dakopatts, Copenhagen, Denmark). They were washed twice with PBT and the enzyme activity was visualized with diaminobenzidine. All steps were performed at room temperature (de Jong et al. 1993).

Immunogold labelling

Cells of the cell line AP284 were collected by centrifugation and fixed for 1 h at 4°C in 0.1 M phosphate buffer (PB), pH 7.3, containing 1% acrolein (Sigma, St.Louis, Mo., USA) and 0.4% glutaraldehyde (grade II, Polyscience, Warrington, USA). Cells were rinsed twice with 0.1 M PB, resuspended in 10% (w/v) gelatin in 0.1 M PB at 37°C, and centrifuged. The pellet was solidified on ice and processed as described previously (de Jong et al. 1993).

BCG infection of mice

Mice were intravenously injected with 6×10^6 viable BCG (strain 601A) suspended in PBS, pH 7.4. Twenty-one days after infection, the spleen, lung and liver were removed and processed for immunostaining according to de Jong et al. (1993).

Enzyme-linked immunosorbant assay

The expression of the ER-HR3 antigen by macrophage cell lines was quantitatively assessed by means of a micro-enzyme-linked immunosorbent assay (ELISA) (Leenen et al. 1990). In short, cells were coated on Terasaki trays with 0.05% glutaraldehyde, incubated for 1 h with ER-HR3, rinsed with PBT, incubated for 1 h with optimally diluted β-galactosidase-coupled anti-rat Ig, rinsed again, and finally incubated for 1 h at 37°C with the fluorogenic substrate 4-methylumbelliferyl galactopyranoside. The amount of generated fluorescent product was then determined with a scanning microfluorometer. The fluorescent product generated in a Terasaki tray saturated with purified rat Ig was used as a positive internal control. Antigen expression was denoted in arbitrary units (AU), implying that, for 100 AU, a fluorescence signal was obtained that equalled this positive control.

Bone marrow cultures

Macrophages were cultured from murine bone marrow under the following conditions. Bone marrow was flushed from femurs with a 23-gauge needle and collected in 6 ml α-medium containing 5% FCS. The cells were centrifuged and resuspended in α-medium supplemented with 10% FCS and 20% L-cell-conditioned medium, the latter as source of monocyte colony-stimulating factor (M-CSF). The cell concentration was adjusted to 5×10^4 cells/ml medium, and 1-ml aliquots were plated in 24-well plates (Costar, Cambridge, Mass., USA) on Lux polystyrene coverslips (Lux, Naperville, Ill.,

USA) and cultured at 37°C in a humidified atmosphere consisting of 5% CO_2 in air. Both the non-adherent and adherent cell fractions were collected every two days until day 12 of culture; the cells were immunostained as described by Leenen et al. (1987).

Antigen presentation

The antigen presentation assay was performed in vitro as described by Klasen et al. (1988). In short, an methylated bovine serum albumine-specific T cell clone was cultured for three days in serum-free medium with or without antigen together with irradiated macrophages. Proliferation of the T cells was measured by ^{3}H-thymidine incorporation during the last 8 or 18 h of culture. The effect of the ER-HR3 antibody in this assay was investigated by addition of increasing amounts of the mAb to the culture medium.

PHZ treatment

Mice were intraperitoneally injected with 1 mg PHZ (Merck, Darmstadt, Germany) dissolved in PBS, on four subsequent days. Three days after the last injection, some mice were intravenously injected with carbon particles 30 min before the removal of their spleens and livers. These organs were removed, sectioned and immunostained with the ER-HR3 monoclonal antibody. Carbon injection and immunostaining were performed as previously described (de Jong et al. 1993).

Results

Purification of the ER-HR3 antigen

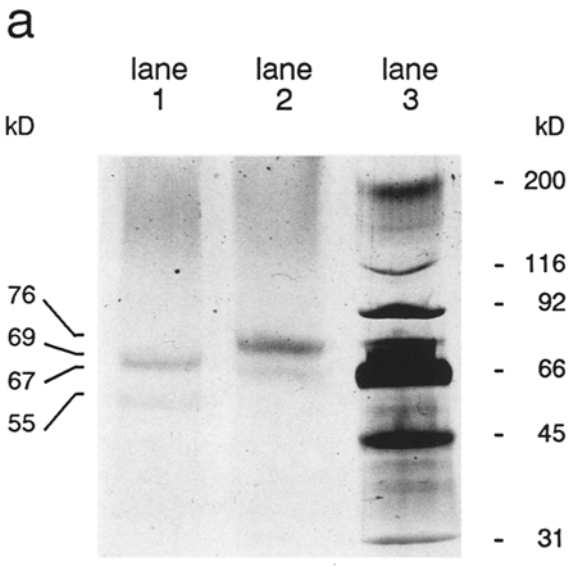
The first part of this study (de Jong et al. 1993) demonstrated that the spleen and the terminal ileum were sites at which ER-HR3-positive cells were abundantly present. We used tissue from these organs to prepare cellular lysates in order to determine the molecular mass of the antigen recognized by ER-HR3. In addition, a cellular lysate of the macrophage cell line AP284 was used. After affinity purification, samples were analysed by SDS-PAGE. Under non-reducing conditions a major protein band with an apparent molecular mass of 69 kDa and a minor band at 55 kDa were observed (Fig. 1a, lane 1). Under reducing conditions, their apparent molecular mass was estimated as 76 kDa and 67 kDa, respectively (Fig. 1a, lane 2). Only the largest of these proteins was present in the culture supernatant of cell line AP284 (not shown). Electrophoretic transfer of the proteins to nitrocellulose and successive incubation with ER-HR3 did not result in immunostaining of the protein bands, irrespective of whether the proteins were reduced or not. However, direct spotting of the unreduced purified proteins on nitrocellulose and successive immunostaining revealed positive spots (Fig. 1b).

Intracellular distribution of the ER-HR3 antigen

The intracellular distribution of the antigen was investigated by immunogold labelling of the cell line AP284. Some of the antigen is present on the outer cell membrane (Fig. 2a). Moreover, most of the antigen present in the cytoplasm is membrane-associated (Fig. 2a). The antigen is also stored in vesicles (Fig. 2b,c).

The ER-HR3 antigen as a macrophage differentiation marker

The question of whether the antigen recognized by ER-HR3 was a differentiation marker was approached in two ways. First, we determined the number of positive cells in ageing M-CSF-stimulated bone marrow cultures. At the initiation of the culture, 50% of the total cell population possessed the ER-HR3 antigen. In time, their number increased and, at day 10 of culture, all cells expressed the marker (Fig. 3a). Secondly, we investigated the expression of the antigen on distinct macrophage lines. These lines could be ordered in a linear maturation sequence



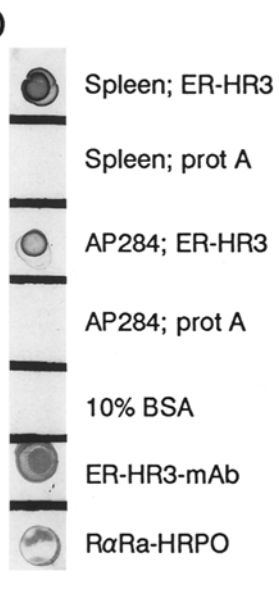


Fig. 1. a SDS-PAGE pattern of a cellular lysate from the spleen after affinity chromatography with the ligand ER-HR3. *Lane* 1, Non-reduced; *lane* 2, reduced; *lane* 3, marker proteins. **b** Spot test of protein A and ER-HR3 affinity-purified cellular lysates from the spleen and the cell line AP284. *Positive spots* were observed only in protein fractions obtained after ER-HR3 affinity chromatography (*Spleen*; *ER-HR3* and *AP284*; *ER-HR3*). Non-specific binding of ER-HR3 to protein A-affinity purified proteins was not observed (*Spleen*; *prot A* and *AP284*; *Prot. A*). BSA (10% *BSA*) was used as a negative control. ER-HR3 (*ER-HR3-mAb*) and the horseradish peroxidase-conjugated rabbit-anti-rat immunoglobulin (*R*αRa-HRPO) were used as positive controls

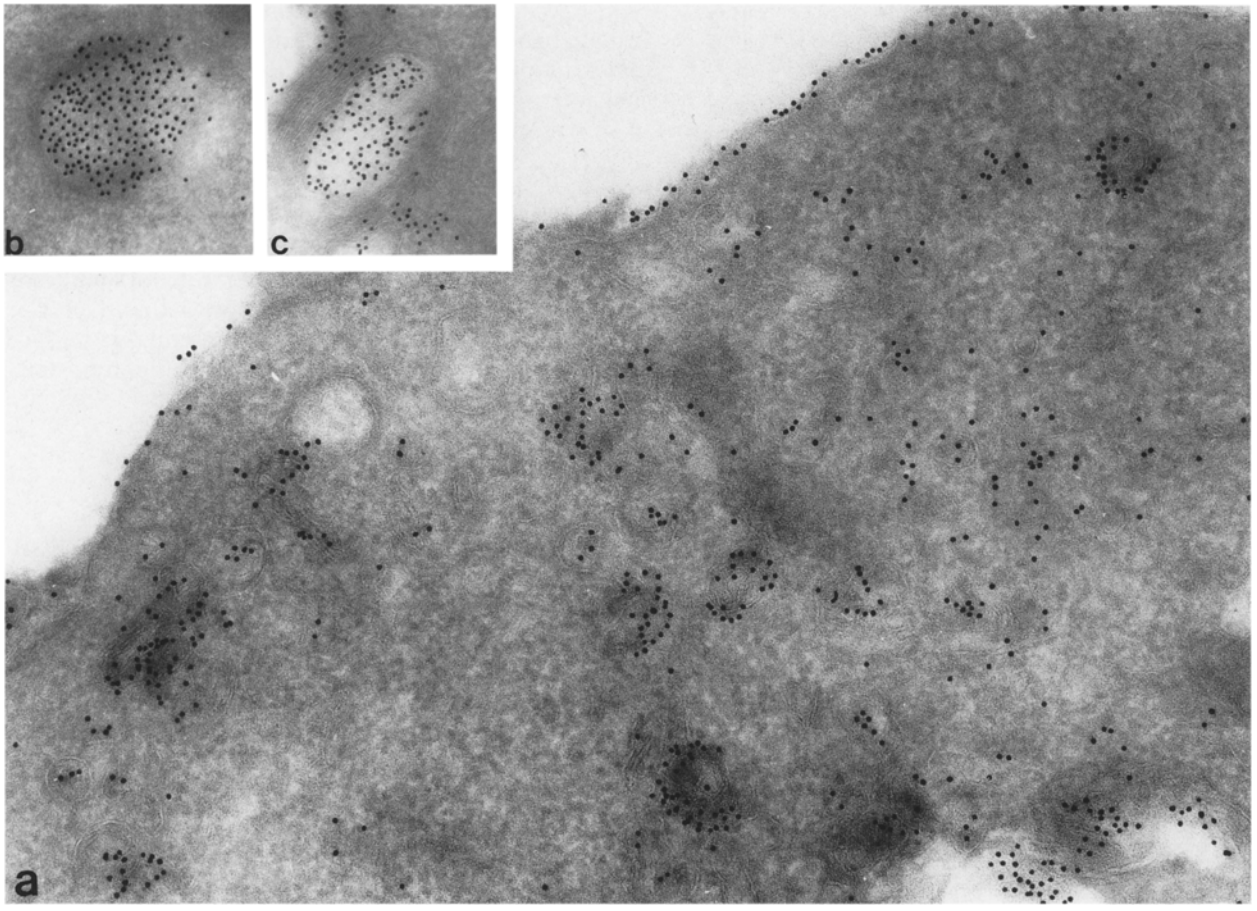


Fig. 2a–c. Intracellular distribution of the ER-HR3 antigen in the cell line AP284. Gold particles are present on the plasma membrane and cytoplasmic membranous structures **(a)** or within vesicles **(b,c)**. × 60 000

depending on the antigens that they expressed (Leenen et al. 1986). The ER-HR3 antigen was expressed by many of these cell lines. In general, lines with a more mature phenotype tended to show a higher expression of the antigen (Fig. 3b) Hence, in both models, expression of the ER-HR3 antigen appeared to correlate with macrophage maturity.

Expression of the ER-HR3 antigen in BCG-infected mice

At day 21 of BCG infection, we investigated ER-HR3 antigen expression by cells in the lung, liver and spleen. For a comparison, we also judged the expression of the CR3 antigen (Mac-1; mAb M1/70), MHC class II antigens (Ia; mAb M5/114) and F4/80 antigen. Control lungs of uninfected mice contained only a few cells positive for F4/80, M1/70 and M5/114. ER-HR3-positive cells were not present in the lungs. Within BCG-infected lungs, granulomata were mainly located near to the bronchi and large arteries. M5/114 staining was not restricted to these sites but was observed throughout the lung (Fig. 4a). Within the granulomata, most cells were heavily stained by M1/70 (Fig. 4c). Macrophages within the lesions showed weak expression of F4/80 (Fig. 4b). ER-HR3 antigen expression was restricted to cells present in the granulomata around the arteries but not in the bronchi (Fig. 4d).

In control livers, most, if not all, Kupffer cells stained with monoclonal antibody F4/80. Approximately 10% of these cells were Ia-positive. The ER-HR3 and Mac-1 antigens were not expressed by cells in the liver. In BCG-infected livers (Figs. 4e–h), the Ia antigen was expressed by many cells, i.e., the sinusoid lining cells, Kupffer cells and by most cells present in the granulomata (Fig. 4e). The F4/80 antigen was present on macrophages in the granulomata and on Kupffer cells (Fig. 4f). Expression of the F4/80 antigen on the latter cell type was strongly increased when compared with uninfected livers. The markers Mac-1 and ER-HR3 were mainly confined to the granulomata (Fig. 4g,h). ER-HR3 staining was less intense than M1/70. In control spleens, F4/80 and ER-HR3-positive cells were confined to the red pulp. M1/70 strongly stained the granulocytes and weakly stained some large macrophages in this region. Moreover, the antigen appeared to be expressed by cells in the marginal zone. Ia expression was restricted to cells in the follicle centre and to some cells in the red pulp. In BCG-infected spleens, the architecture was disrupted by the formation of granulomata. The region surrounding the marginal zone was especially affected. Infiltrated cells weakly expressed the antigens recognized by ER-HR3 and F4/80.

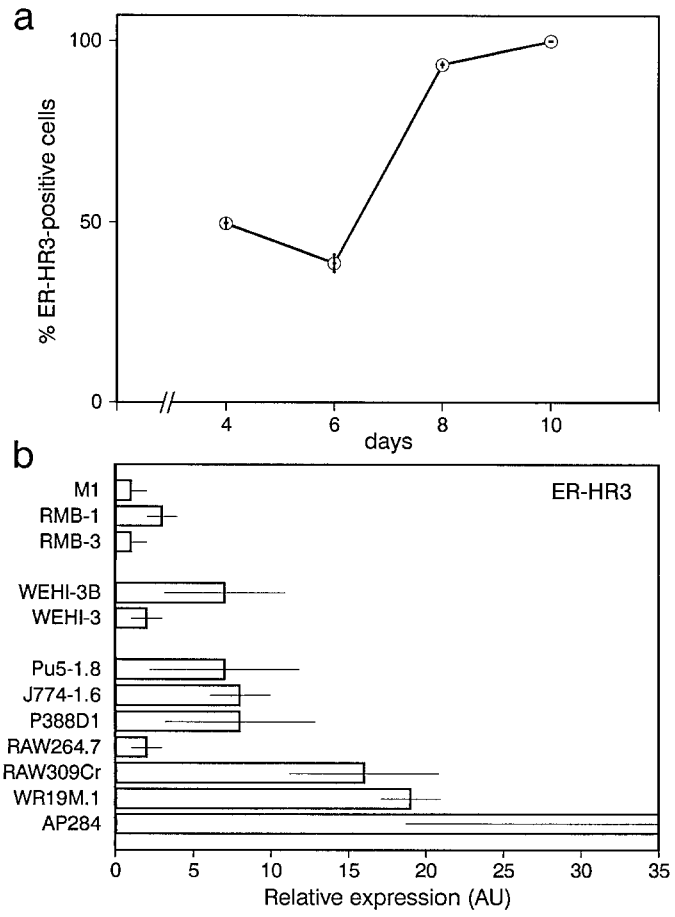


Fig. 3. a ER-HR3 and F4/80 antigen expression on M-CSF-stimulated bone marrow cells. Data are presented as a percentage of the total cell population. **b** ER-HR3 antigen expression by macrophage cell lines. These are arranged in order of maturation according to Leenen et al. (1986). *M1* exemplifies the most immature and *AP284* the most mature cell line

The Ia and the Mac-1 antigen were both strongly expressed on cells throughout the spleen.

Expression of the ER-HR3 antigen in PHZ-treated mice

Mice injected with PHZ are known to develop haemolytic anemia. They show splenomegaly and extramedullary erythropoiesis in the liver. In PHZ-treated mice, the splenic structure was disrupted because of a marked enlargement of the red pulp. The increase in erythropoietic activity was readily visualized because of the presence of many erythroblasts. Moreover, there was a large increase in the number of ER-HR3-positive cells (Fig. 5a) in the spleen. Positive cells appeared to be larger compared with those in control mice. They were often surrounded by erythroid precursors (Fig. 5b). The most striking difference between control and PHZ-treated mice with respect to the distribution of the ER-HR3 antigen was the appearance of ER-HR3-positive cells in the liver (Fig. 5c). They were mainly present in the liver sinusoids. Occasionally, they were surrounded by a number of erythroblasts (Fig. 5c, insert). Injection of carbon after PHZ treatment resulted in carbon staining of all ER-HR3-positive cells of the liver, including the central macrophages surrounded by erythroblasts (Fig. 5d).

Antigen presentation

ER-HR3 antibody had no effect on mBSA presentation by the ER-HR3- -positive cell line AP284 to mBSA primed T cells (data not shown). However, mAb M5/114 (anti-Ia), used as a positive control, greatly diminished antigen-specific T cell proliferation.

Discussion

In a previous paper, we have reported the distribution of the antigen recognized by an antibody ER-HR3 that is directed against a subpopulation of macrophages located predominantly in lympho- -haemopoietic organs (de Jong et al. 1993). Here, we give details concerning the purification of the antigen recognized by ER-HR3 and its expression under different experimental conditions. ER-HR3 recognizes two proteins with apparent molecular masses of 69 kDa and 55 kDa, respectively (Fig. 1b). The observation that these molecular masses increase under reducing conditions is indicative of the presence of intramolecular disulphide bridges. Since both proteins share this property, they may be related in structure. The intracellular distribution of the antigen, i.e., membrane-associated and vesicle-stored (Fig. 2), together with the isolation of only the 69 kDa protein from the supernatant of a monocytic cell line favour the hypothesis that the antigen is present in a membrane-associated and in a soluble form, a phenomenon also observed for IL-1 (Kurt-Jones et al. 1985). The membrane-associated occurrence of the antigen correlates well with observations from our in vivo study (de Jong et al. 1993). However, macrophages in situ lack the antigen-loaded vesicles as observed in AP284 cells. The assumption that the secretion rate of the antigen under in vivo conditions is much higher, preventing the storage of the protein in the vesicles, may explain this observation.

The data from our differentiation studies indicate that the antigen recognized by ER-HR3 is probably expressed by the more mature cells of the mononuclear phagocyte system. In cultures of M-CSF-stimulated bone marrow, the number of ER-HR3-positive cells increases gradually with time (Fig. 3a). Additionally, when distinct macrophage cell lines are arranged in a differentiation sequence (Leenen et al. 1986), the antigen is predominantly present on cell lines with a more mature phenotype (Fig. 3b). For example, cells of the AP284 cell line possess a number of macrophage-specific antigens, are able to phagocytose carbon and stain positively for the enzymes acid phosphatase and non-specific esterase (Klasen et al. 1988).

To obtain more information about the functional properties of the antigen, we have investigated its expression under different experimental conditions, i.e., BCG infection and PHZ treatment. In BCG-infected mice, the

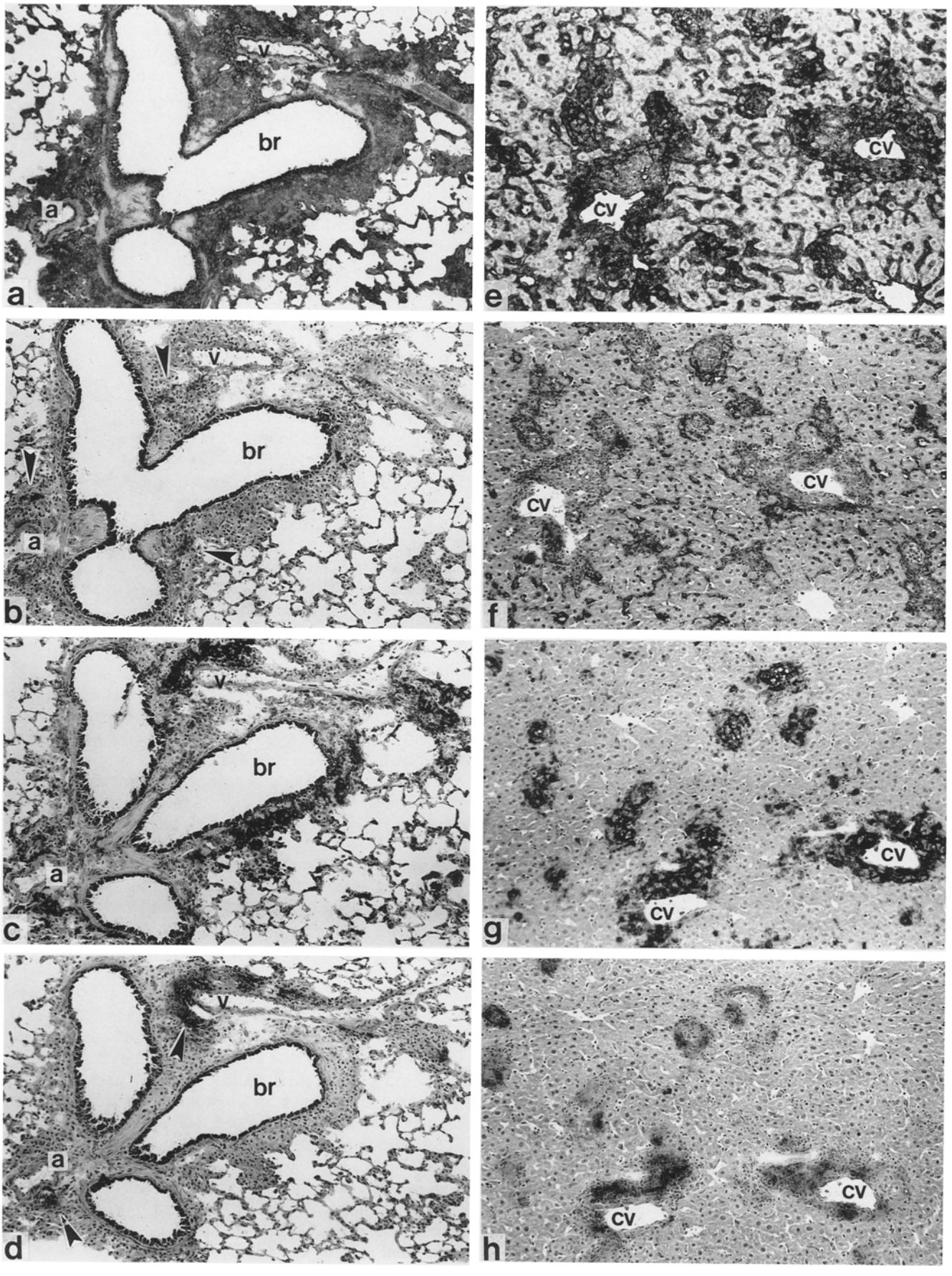


Fig. 4a–h. Distribution of macrophage antigens in the lung **(a-d)** and liver **(e-h)** of BCG-infected mice. Antigens investigated were Ia **(a,e)**, F4/80 **(b,f)**, Mac-1 **(c,g)**, and ER-HR3 **(d,h)**. *Arrowheads* **(b, d)** indicate the sites of positive staining. × 60. *a* Artery; *br* bronchiolus; *cv* central vein; *v* vein

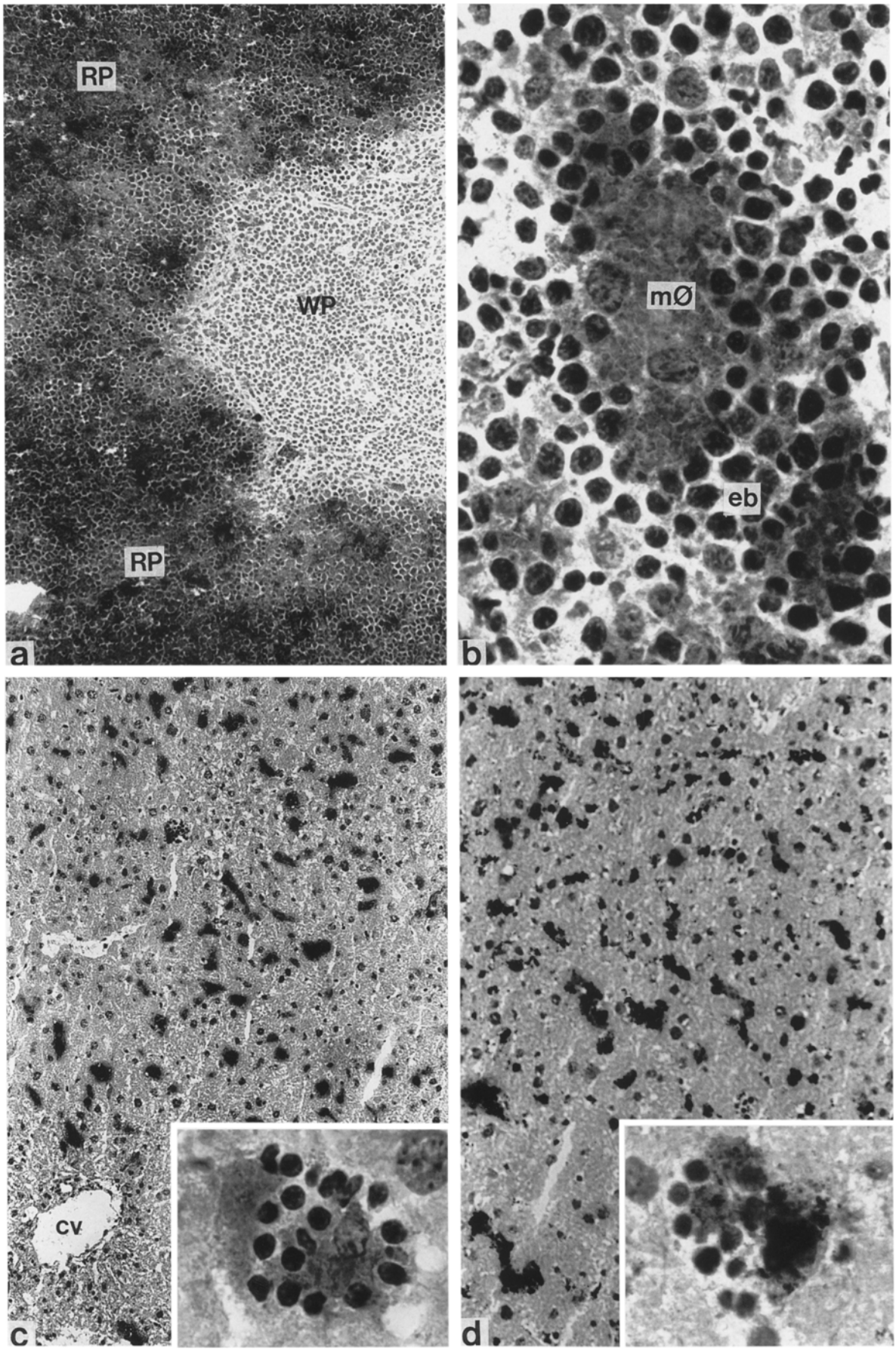


Fig. 5a–d. Distribution of ER-HR3-positive cells in the spleen **(a,b)** and liver **(c,d)** of PHZ-treated mice. **a** ER-HR3-positive cells are abundant in the red pulp. × 125. **b** High-power magnification of erythrocyte-loaded ER-HR3-positive macrophages (*mØ*) surrounded by erythroblasts. × 650. **c** Distribution of ER-HR3-positive cells in liver. × 140. *Insert* High-power magnification of ER-HR3-positive cell surrounded by erythroblasts. × 650. **d** Distribution of carbon-loaded cells in the liver. *Small spots* indicate Kupffer cells; *large spots* indicate carbon-loaded ER-HR3-positive cells. × 140. *Insert* High-power magnification of a carbon-loaded ER-HR3-positive cell surrounded by erythroblasts. × 650. *cv* Central vein; *eb* erythroblasts; *RP* red pulp; *WP* white pulp

number and size of granulomata in the various organs reach maximal values at day 21 (Nibbering et al. 1989). Differences in the localization and expression of the distinct macrophage antigens under these conditions are most profound in the liver (Fig. 4). Ia and F4/80-positive cells are present throughout the organ. In contrast, Mac-1 and ER-HR3-positive cells are confined to the granulomata. These latter macrophages are possibly recruited from other sites. Such recruitment of Mac-1-positive cells has also been described after infection of mice with *Plasmodium yoelii* or *Listeria monocytogenes* (Gordon et al. 1988). However, it is also possible that pro-inflammatory cytokines, present in the granulomata, induce antigen expression on local macrophages. The observation that ER-HR3-positive cells are exclusively present in granulomata around the pulmonary arteries, but not the bronchi, of BCG- -infected mice favours the hypothesis that only the migratory macrophages express this marker. Other studies with infectious agents indicate that the expression of MHC class II antigens is correlated with macrophage activation (Ezekowitz et al. 1981; Adams and Hamilton 1987). In our study, we have demonstrated that the expression of ER-HR3 and Ia does not co-exist. Therefore, it is unlikely that the ER-HR3 antigen is involved in the process of activation.

ER-HR3 antigen expression has also been investigated after PHZ injection. In adult mice, repeated injections of PHZ cause haemolytic anemia that is accompanied by extramedullary erythropoiesis in the liver (Ploemacher and van Soest 1977). Interestingly, after PHZ treatment, many cells in the liver are stained positively by ER-HR3 (Fig. 5). They occur in the liver sinusoids and veins. Some of them are surrounded by a rim of erythroblasts and are considered to be central macrophages. Carbon injection after PHZ treatment results in carbon labelling of all ER-HR3-positive cells, including the central macrophages. Although an influx of macrophages from other sites cannot be excluded, this result suggests that PHZ treatment induces ER-HR3-expression on a subpopulation of Kupffer cells. The observation that Kupffer cells under anoxic conditions are able to produce erythropoietin (Paul et al. 1984), together with our observation that ER-HR3-positive Kupffer cells are surrounded by erythroblasts, argues for a possible role of the ER-HR3 antigen in erythropoiesis. Moreover, the antigen is expressed by renal medullary interstitium cells (de Jong et al. 1993); under physiological conditions, erythropoietin is produced at this site (Schuster et al. 1987). Other indications for the presumed function of the ER-HR3 antigen in erythropoiesis are the occurrence of erythropoietic islands with ER-HR3-positive central macrophages in the fetal and adult spleen, the fetal liver and adult bone marrow (de Jong et al. 1993). Nevertheless, ER-HR3-positive cells also occur at other locations and it cannot be excluded that the antigen is involved in other processes. For example, the presence of ER-HR3-positive cells in the T-cell-associated regions of lymph nodes may be indicative of a function in the immune response. Our observation that ER-HR3 is unable to inhibit antigen-specific T cell proliferation makes it unlikely that the ER-HR3 epitope is directly involved in antigen presentation.

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