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Journal of Immunological Methods 275 (2003) 123–132

JIM
Journal of
Immunological Methods

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Analysis of intra-hepatic peptide-specific cell recruitment in mice immunised with *Plasmodium falciparum* antigens

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Received 21 June 2002; received in revised form 8 November 2002; accepted 6 January 2003

Abstract

The liver stage of *Plasmodium* spp. now appears as a relevant target of immune effectors triggered by the so-called "anti-sporozoite" vaccine. Since the monitoring of immune responses at the systemic level may not faithfully reflect the local protective mechanisms, the aim of the present work was to set up a model to study the local intra-hepatic cellular responses and to compare these with the peripheral immune responses. This was achieved by intra-portal delivery of epitopic peptides, i.e. peptides containing B and T cell epitopes, which were coated onto the surface of polystyrene microbeads. The peptide-coated beads presumably mimic the hepatic schizont, and when distinct peptides are administered separately, this method of delivery allows us to decipher the immune responses resulting in mice immunised with recombinant proteins spanning several such epitopes. Using the *P. falciparum* liver stage antigen-3 (LSA3) molecule, which can induce protection against a sporozoite challenge, our results show that 25-μm microbeads could easily access the liver parenchyma by intra-portal injection and were distributed evenly in the liver. Also, LSA3-derived synthetic peptides coated onto microbeads initiated specific cell recruitment within 6 h. Depending on the LSA3 peptide used, the infiltrates induced differed in size, with the strongest cell recruitment obtained using nonrepeat II peptide (NR2)-coated microbeads with a mean leukocyte number of 79 per granuloma. Immunohistological studies of liver sections revealed that, irrespective of the delivered peptide, cells infiltrating the liver towards microbeads were mainly $CD3^+$ T lymphocytes, both $CD4^+$ (70 to 80%) and $CD8^+$ (20 to 30%) subtypes, macrophages and dendritic cells. Cells infiltrating the granuloma had features of activated cells, with evidence of VLA-4 cell-surface expression, and production of IFN-γ and IL-4. Analysis of the peripheral B and T-cell responses in the same animals revealed that, whereas the local responses were directed mainly towards NR2 and repeat peptides (RE), the peripheral T-cell response to these peptides was weak and infrequent, although antibody production was high.

Keywords: *Plasmodium falciparum*; Liver stage antigen-3; Local immunity; Lymphocyte migration; Peptide; Microspheres

Abbreviations: LSA3, liver stage antigen-3; NR1, nonrepeat I peptide; NR2, nonrepeat II peptide; RE, repeat peptide; GST, glutathione-*S*-transferase; LMC, lymphomonocytic; CTL, cytotoxic T lymphocyte.

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doi:10.1016/S0022-1759(03)00017-6

1. Introduction

Although the liver stages of *Plasmodium* were discovered over 50 years ago, it has only recently become clear that they are the target of in vivo parasite-specific protective immune mechanisms (for review, see Druilhe et al., 1998). Of the large number of immune effectors, mainly specific for the circumsporozoite protein (CSP), which have been characterised in vitro against these stages (Chen et al., 1977; Schofield et al., 1987; Hoffman et al., 1989; Renia et al., 1991, 1993), it remains paradoxically unclear which one(s) may play a protective role. A major reason is the lack of appropriate immunological tools for evaluating the efficacy of local immune responses to discrete parasite molecules or peptides. Indeed, vaccine development is generally based on the assessment of immune effectors present in peripheral blood cells, whereas cellular events in the liver are likely to be of greater relevance. This view is supported by studies in which, out of several *Plasmodium yoelii*-specific $CD8^+$ cytotoxic T lymphocyte (CTL) clones with similar in vitro cytotoxic activity, only a few have been found protective against sporozoite challenge, as shown by adoptive transfer experiments (Rodrigues et al., 1992). Similar conclusions were drawn by Vanderberg and co-workers who observed infiltrated inflammatory cells around *P. yoelii* liver schizonts in challenged mice after immunisation with attenuated *P. yoelii* sporozoites (Khan and Vanderberg, 1992) or even with sporozoites of *Plasmodium berghei* (Vanderberg et al., 1993).

We have developed a new model aimed at investigating the local intra-hepatic responses through the use of polystyrene beads which permits the hepatic delivery of liver stage antigens, particularly individual epitopes. The use of this model also facilitated the comparison between the local intra-hepatic and the peripheral immune responses. This work has focused on the *P. falciparum* liver stage antigen-3 (LSA3) pre-erythrocytic stage antigen and its derived synthetic peptides (BenMohamed et al., 1997; Perlaza et al., 1998; Daubersies et al., 2000), primarily because LSA3 has been shown to induce protection in chimpanzee against a *P. falciparum* (Daubersies et al., 2000) and in mice against a *P. yoelii* challenge (Brahimi et al., 2001).

2. Material and methods

2.1. Recombinant protein and synthetic peptides

The LSA3-derived 729-S recombinant protein fused to glutathione-*S*-transferase (GST-729-S) was produced in *Escherichia coli* and purified on glutathione as described previously (Daubersies et al., 2000).

Two 26-mer peptides from the nonrepeated region (NR1 and NR2) and one 28-mer peptide from the repeated region (RE) of the 729-S fragment (Table 1) were chosen on the basis of their structural properties encompassing hydrophobic residues and defining strong T cell and B cell epitopes in mice, chimpanzees and humans (BenMohamed et al., 1997). The CT1 peptide located outside the recombinant 729-S sequence was used here as a negative control. All peptides were synthesised as described previously (BenMohamed et al., 1997).

2.2. Passive adsorption of antigens to polystyrene beads

Polystyrene beads of 0.5 μm were used for immunisation without adjuvant, and beads of 25 μm were used for visualisation of the intra-hepatic peptides. Both the immunogen, the GST-729-S fragment and the synthetic peptides used for in situ analysis were passively adsorbed onto synthetic polystyrene monodispersed microparticles (Polysciences, WA, USA). Microparticles, 0.5 μm, were employed for immunisation and 25-μm particles (referred to as microbeads) for in situ studies.

Table 1
The synthetic peptides of the central (729-S) region of LSA3 used in this study

Peptide	Amino-acid sequence	Localization in the protein
LSA3-CT1	L L S N I E E P K E N I I D N L L N N I	24–43
LSA3-NR1	D E L F N E L L N S V D V N G E V K E N I L E E S Q	60–85
LSA3-NR2	L E E S Q V N D D I F N S L V K S V Q Q E Q Q H N V	81–106
LSA3-RE	V E S V A P S V E E S V A P S V E E S V A E N V E E S V	183–210

GST-729-S-coated microparticles were obtained by incubating 1.8×10^{10} particles (0.5-μm diameter) with 50 μg of the recombinant protein overnight at 4 °C in glycine buffer (20 mM glycine, 0.15 M NaCl, pH 8.4), followed by three washings in PBS pH 7.4. Coated microparticles were resuspended in PBS, pH 7.4, before injection. The efficacy of coating was controlled by an agglutination assay using human serum obtained from an individual living in a malaria endemic area (and containing high anti-729-S specific ELISA antibody titers). Absorbance (280 nm) measurement of the protein concentration before and after bead coating revealed that 70% to 80% of the protein was adsorbed onto microparticles.

Peptide-coated microbeads were obtained by incubating 5.6×10^4 particles (25-μm diameter) with 50 μg of one of the four individual peptides, CT1, NR1, NR2 or RE, in glycine buffer, and resuspended in PBS, pH 7.4, before injection. In this case, the efficacy of coating was examined by ELISA measurement of peptide concentration before and after adsorption using sera from mice immunised with the corresponding peptides.

2.3. Immunisation of mice

Six-week-old C3H/HeJ (H-2^k) mice were purchased from C.E.R Janvier (Le Genest, France). Mice were primed subcutaneously with 3.6×10^{10} microparticles coated with 50 μg of recombinant protein GST-729-S resuspended in 200-μl PBS, without adjuvant. Three weeks later, they were boosted under the same conditions with the same number of microparticles.

2.4. Intra-portal injection

Twenty days after the second injection, mice were anaesthetised by subcutaneous injection of 50 μl of a 5 mg/ml solution of Valium (Roche, France) followed by 150 μl of a 20 mg/ml solution of ketamine (Imalgene 500®, Merial, Lyon, France). After median laparotomy, the portal vein was isolated to facilitate the introduction of a 27-G fine needle (BD, Le-Pont-de-Claix, France). Peptide-coated microbeads (6×10^4 in 200 μl RPMI/mouse) were then injected slowly into the portal vein through a 0.4-mm-diameter catheter (Microflex, Vygon, Belgium). A hemostatic gaze (Surgicel 2, Ethicon) was applied to the site of injection and the abdominal wall was sutured. For each of the four peptides, at least five mice were injected.

2.5. Tissue fixation

Two days following intra-portal injection, the mouse liver was removed, cut into 3- or 4-mm fragments which were then fixed and sectioned as follows. In some experiments the livers were collected 6 h, 12 h, or 5 days after intra-portal injection.

Two alternative protocols of tissue fixation were used depending on the immunostaining used thereafter: (A) Cryopreservation by embedding tissues in Tissue-Tek® O.C.T. Compound (Sakura, Zoeterwoude, The Netherlands) and bringing them to −80 °C; (B) chemical fixation using a zinc solution instead of classical organic solvents as the latter can alter some of the surface antigens studied, followed by alcohol dehydration. The fixed tissues were then immersed twice in a low-temperature paraffin for 3 h under vacuum (37 °C melting point polyethyleneglycol distearate, Aldrich Chemical, Milwaukee, WI, USA). A final overnight paraffin bath was used to orientate the liver biopsy. The blocks were stored at +4 °C until immunochemical analysis was performed.

For frozen specimens, serial 5-μm sections were cut using a cryostat (HM 500 OM, Microm, Francheville, France) at −21 °C. These were placed directly onto precleaned silaned slides (SuperFrost/Plus®, Menzel-Glaser, Germany), allowed to dry at room temperature, fixed in acetone and kept at −80 °C until immunostaining. Paraffin blocks were brought to +4 °C before sectioning using a rotary microtome (2055 AUTOCUT, Reichert-Jung/Leica Microsystems, Wetzlar, Germany). Serial 5-μm sections were transferred from water at 37 °C onto silaned glass as above. Paraffin was removed by immersion in ethanol, and the slides were dried at room temperature and kept at 4 °C until immunostaining.

2.6. Histochemistry

To detect the presence of liver cell infiltrates, frozen and PEG-dewaxed sections were stained with haematoxylin–eosin before mounting in Eukitt

medium. The quantification of granulomas and cells was performed on 1 every 10 serial 5-μm sections to avoid counting the same cell granuloma twice (microbeads being of 25-μm diameter, the probability of seeing the same granuloma twice had to be taken into account). A mean of 50 granulomas was analysed for each peptide.

2.7. Immunocytochemistry

Both cryo-cuts and paraffin-embedded sections were rehydrated in Tris–buffer saline 0.05 M pH 7.6 (TBS). The slides were washed twice in TBS between each step of the procedure. After a saturation step with normal rabbit serum diluted 1:10, sections were incubated (90 min at RT) with a primary rat antibody diluted in TBS + 1% milk, followed by incubation with a phosphatase-labelled rabbit anti-rat IgG (Dako, Z 0455) for 45 min. The primary antibodies used were rat monoclonal antibodies to mouse CD3 (MCA500G, IgG2a, Serotec, Oxford, UK), CD4 (RM2500, IgG2a) and CD8 alpha (RM2200, IgG2a) (Caltag Laboratories, Burlingame, CA), macrosialine, a cell surface antigen of macrophages as well as dendritic cells (FA11, IgG2a), H-2 I-$E^{d,k}$ (M5/114, IgG2b), IL-4 (11B11, IgG1) and IFN-γ (R4-6A2, IgG1), purchased from American Type Culture Collection (ATCC; Rockville, MD). The APAAP complex (alkaline phosphatase-labelled rat monoclonal anti-alkaline phosphatase, Dako D 0488), diluted 1:50, was then added for 30 min. Following washes, the enzymatic activity was revealed by adding the FAST RED substrate which was prepared by mixing 14.4 mg of levamisole and 50 mg of FAST RED-TR salt in 50 ml of TBS 0.1 M pH 8.2 containing 10 mg Naphthol-As-Mx (3-hydroxy-2-naphtoic acid 2,4-dimethylanilide) phosphate, and 1 ml dimethyl-formamide, all purchased from Sigma, St Louis, USA. The reaction was followed under the microscope until the appearance of staining, and stopped by adding water. The sections were counter-stained with Shandon hematoxylin before mounting in an Immunomount medium.

2.8. T-cell proliferation assays

Spleen cell suspensions were obtained from each spleen, and the cells were washed twice in RPMI 1640 and then resuspended at a concentration of 5×10^6 cells/ml in RPMI supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, 50 μM β-mercaptoethanol, 1.5% foetal calf serum and 0.5% normal mouse serum. Aliquots of 100 μl of cell suspension were distributed into round-bottomed 96-well microculture plates (Costar) and peptides added at the indicated concentrations. Cultures were set up in triplicate. Following 48-h culture (37 °C and 5% CO_2), 50 μl/well of supernatant was collected and stored at −70 °C for IFN-γ titration. [^{3}H]Thymidine (Amersham Life Science, UK) was added (1 μCi/well) during the last 6 h of a 72-h incubation period. The cells were harvested using an automatic cell harvester (Skatron, Sterling, VA, USA) and [^{3}H]thymidine incorporation was quantified by scintillation counting.

2.9. IFN-γ titration

IFN-γ in T-cell supernatants was titrated by a two-site capture ELISA. Flat-bottomed 96-well plates (Maxisorp, Nunc) were coated overnight at 4 °C with a primary rat anti-mouse IFN-γ (R4-6A2) mAb (Pharmingen, San Diego, CA), diluted in 0.1 M, pH 9.6, Na_2CO_3 buffer. Between each step of the procedure, plates were washed several times with PBS + 0.05% Tween. Plates were then saturated with a 3% BSA (Sigma) solution in PBS + 0.05% Tween (PBT). Undiluted supernatants were added to precoated wells and incubated overnight at 4 °C, followed by 1-h incubation at RT with a biotinylated secondary rat anti-mouse IFN-γ (XMG1.2) mAb (Pharmingen), diluted in PBT. Following steps of peroxidase-labelling, staining and reading were identical to the final procedure of mouse antibody detection by ELISA described above.

3. Results

3.1. Peripheral immune responses to the LSA3 peptides

Immune responses in peripheral blood cells were evaluated in mice after immunisation and before intraportal peptide delivery, using conventional in vitro assays. Spleens of immunised mice were removed 2

weeks after the second immunisation with the recombinant protein and T-cell responses evaluated by both proliferation and IFN-γ release upon in vitro restimulation of spleen cells with the shorter synthetic peptides. Peripheral proliferative T-cell responses were generally low for all peptides, and showed variations from one mouse to another (Table 2). Indeed, four out of five mice showed a weak response to peptide NR2, and three out of five mice to peptide NR1, suggesting a similar antigenicity for both peptides. In two out of five mice, proliferative responses to both peptides coexisted. No significant responses could be detected to peptide RE. Similarly, IFN-γ was released in high amounts from NR2- (6/8) and NR1- (7/8) stimulated cultures (Table 2), whereas secretion of this cytokine was observed at lower levels against peptide RE in 3/8 mice. INF-γ secretion in response to both peptides NR1 and NR2 was detected in 5/8 mice. In contrast, high antibody responses to peptide NR2, and at a lesser extent to NR1 and RE, were detected by ELISA (data not shown).

3.2. Microparticles access the liver parenchyma following their intra-portal injection

In an attempt to establish a parallelism between the systemic immunological status of mice and their in situ response in the liver, we first optimised the experimental conditions of microparticle delivery into the mouse liver. The influence of the size (25 or 5 μm) and volume of microparticles injected were studied, as well as the type and dose responses to the anaesthetic agent used. The best results were obtained by intra-portal delivery of 5.6×10^4 25-μm microparticles in 200-μl PBS, which caused only moderate trauma in naive mice with most animals surviving. In contrast, 5-μm microparticles frequently induced death less than 24 h post-injection, possibly due to their ability to aggregate and generate local thrombosis resulting in hepatic necrosis. As shown in Fig. 1A, 48 h after delivery into the portal vein, the 25-μm microparticles were readily detectable in the hepatic parenchyma tissue and easily visualised at low magnification in the vicinity of the portal veins from which they had migrated. This result is consistent with the known plasticity of the endothelial membrane, which separates hepatic cells from blood. Microparticles were randomly distributed in the liver, although limited

Table 2
Splenocyte responses to LSA-3 peptides in mice immunised with LSA3 derived GST-729-S

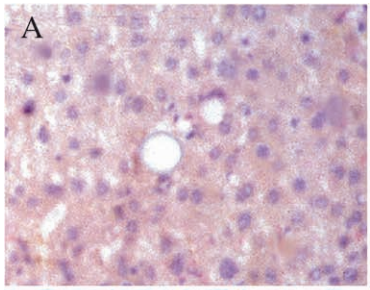
Mouse	In vitro stimulation	Stimulation index (SI)	Delta cpm	IFN-γ release (ng/ml)
1903	NR1	1.5	2031	5
	NR2	2.4	5287	8
	RE	1.3	1492	8
	CT1	1.1	780	6
1507	NR1	1.1	724	0
	NR2	1.5	4267	17
	RE	0.9	− 619	6
	CT1	0.8	− 1500	11
2810	NR1	1.8	21,693	5.8
	NR2	1.0	367	0
	RE	1.1	2502	0.84
	CT1	0.9	− 2950	1
251	NR1	1.8	17,276	6
	NR2	1.8	16,755	18
	RE	1.0	− 1109	10
	CT1	0.6	− 9179	0
253	NR1	1.4	9240	4
	NR2	1.3	7554	8
	RE	1.0	1436	n.d.
	CT1	0.6	− 9305	0
209	NR1	n.d.	n.d.	12
	NR2	n.d.	n.d.	47
	RE	n.d.	n.d.	4.5
	CT1	n.d.	n.d.	1
210	NR1	n.d.	n.d.	6
	NR2	n.d.	n.d.	29
	RE	n.d.	n.d.	0.5
	CT1	n.d.	n.d.	0.5
Pool[a]	NR1	n.d.	n.d.	3
	NR2	n.d.	n.d.	7
	RE	n.d.	n.d.	0
	CT1	n.d.	n.d.	0
Naive[b]	NR1	0.7	− 2403	0
	NR2	0.7	− 2459	0
	RE	0.8	− 1337	0
	CT1	0.8	− 1033	0.1

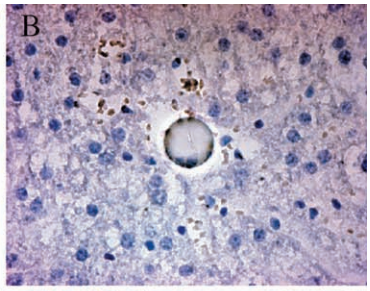
Spleen cells of C3H/Hej mice immunised s.c. with the recombinant protein coated onto microparticles were stimulated in vitro with the synthetic peptides of LSA-3 as described in the Materials and methods. Stimulation indices (SI) and delta cpm values are relative to background cpm (medium alone). IFN-γ secretion was titrated in 48-h culture supernatants and titres were deduced following subtraction of background values. All values shown are the mean of triplicates. Concavalin A-stimulated cultures yielded SI values ranging from 5 to 10, and IFN-γ titers > 100 ng/ml. Experiments were conducted in mice belonging to a different group than those used for immunohistochemistry studies (n.d.: not done).

[a] Spleens from another group of three immunised mice were pooled and this was considered as one group.

[b] Mean of two unimmunised mice tested individually.

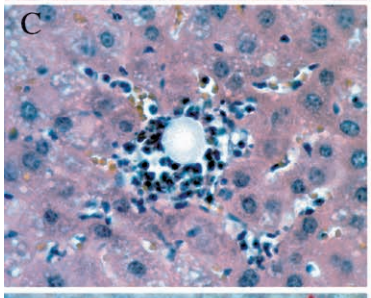
25-μm microparticles not coated with peptides

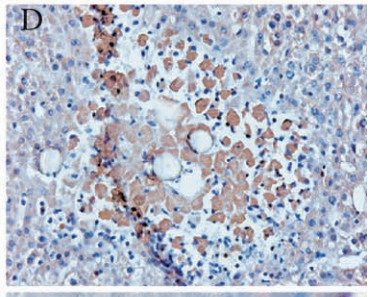




Microparticules coated with the control peptide CT1

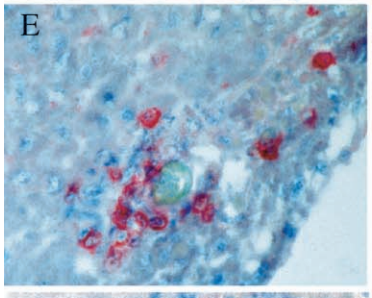
LSA-3 NR2-coated microparticles and lymphomyeloid cells

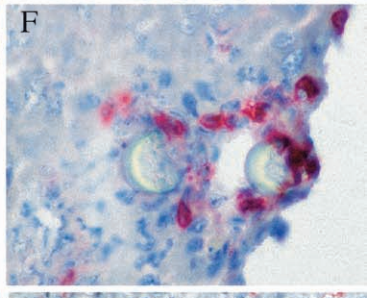




Hepatocellular necrosis near LSA-3 NR2-microparticle-induced granulomas.

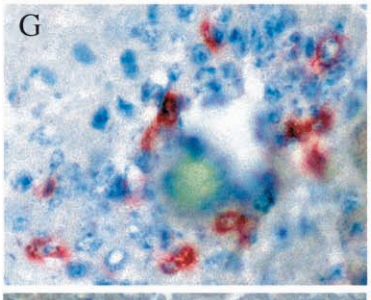
CD3+ within LSA-3 NR2-induced granulomas

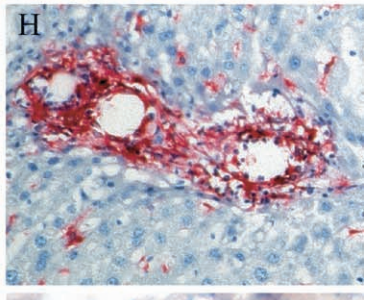




CD4+ within LSA-3 NR2-induced granulomas

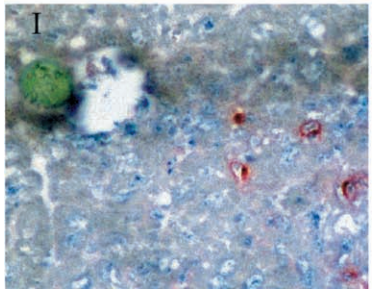
CD8+ within LSA-3 NR2-induced granulomas

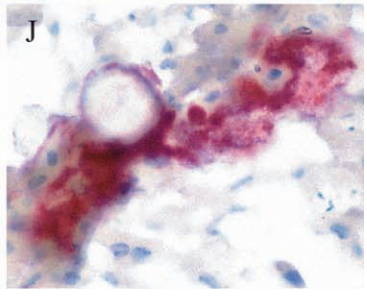




macrophages and Kupffer cells within LSA-3 NR2-induced granulomas

IFN-g secreting cells in NR1- and NR2-induced infiltrates





IL-4 positive cells in response to NR2 peptide

clustering was sometimes observed at the periphery of the hepatic lobule.

3.3. Intra-hepatic delivery induces peptide-specific recruitment of mononuclear cells

To explore the local immunological responses induced by synthetic peptides NR1, NR2, and RE derived from the *P. falciparum* LSA3 antigen, C3H/HeJ mice immunised with the LSA3-derived 729-S recombinant protein were subjected to intra-portal delivery of microparticles coated with either one of these peptides. Our results show that each peptide was able to induce substantial local cell-recruitment 2 days after intra-portal delivery of particles, a time gap sufficient to detect delayed type hypersensitivity (as shown in Fig. 1C for NR2). Haematoxylin staining of liver sections revealed that cell parenchymal infiltrates consisted of lymphomyeloid cells surrounding each microparticle. Liver cell injury around the inflammatory focus, a zone of necrosis surrounded by a ring of pale altered hepatocytes (Fig. 1D), provides indirect evidence of local inflammation and activation of cell recruitment. The extent of such necrosis was correlated with the size of cell granulomas. The maximal zone of necrosis was observed with NR2-coated microparticles, but was absent from control peptide CT1 and appeared intermediate with the remaining peptides. Samples taken at 6, 12 and 48 h post-injection showed that lymphocyte recruitment was already substantial at 6 h and increased over time (not shown). Subsequently, all lymphocyte studies used 48-h samples, since these showed maximal recruitment.

The cell recruitment proved to be antigen-specific since uncoated microparticles (Fig. 1A) or microparticles coated with the control peptide CT1 (Fig. 1B) induced no cell recruitment whatsoever 48 h as well as 5 days after the intra-portal injection. Fig. 1A shows that uncoated beads, despite their relatively large size, were devoid of any intrinsic physical or chemical properties that would potentially generate local inflammation on their own. Moreover, beads coated with peptides NR1, NR2 or RE and injected into nonimmunised mice, or into mice immunised with an irrelevant peptide also, did not induce any cell recruitment at 48 h to 5 days after intra-portal injection (not shown).

3.4. The number of cells recruited varies depending on the peptide

Although each of the three LSA3 synthetic peptides was effective at inducing cell recruitment, the resulting intensities of cell recruitment differed between the peptides. The observed numbers of leukocytes surrounding 50 granulomas per mouse liver from an animal receiving a single type of peptide are shown in Fig. 2. These values point to both an intense and reproducible intra-hepatic response to peptide NR2, in contrast to the peripheral proliferative T-cell responses that were generally low and variable between mice to this peptide. Indeed, the largest infiltrates were observed around the NR2 peptide-coated microparticles (mean number of 59 leukocytes; Fig. 2), followed by those around peptide RE that comprises the repetitive region of LSA3, and then around the peptide NR1 with mean numbers of 39 and 25 leukocytes, respectively.

3.5. Heterogeneity of the cell types recruited

Immunohistological studies of liver sections from mice immunised with 729-S and receiving intra-portal

Fig. 1. Lymphomonocytic cell infiltrates around the intra-portally injected LSA3 peptide coated-microparticles in LSA3-GST-729-S-immunised mice. C3H/HeJ mice were immunised with LSA3-729-PGEX and intra-portally injected with microspheres coated with various synthetic peptides derived from LSA3. The liver was removed 48 h later. (A) Absence of local cell recruitment around 25-μm microparticles not coated with peptides. (B) Microparticules coated with the control peptide CT1. (C) Leukocyte infiltrates induced by the LSA-3 NR2-coated microparticles and showing lymphomyeloid cells surrounding each microparticle. (D) Hepatocellular necrosis is sometimes observed in the vicinity of LSA-3 NR2-microparticle-induced granulomas. Numerous $CD3^+$ (E), $CD4^+$ (F) and $CD8^+$ (G) cells are present within LSA-3 NR2-induced granulomas, but the most representative cells are macrophages and Kupffer cells (H). IFN-γ secreting cells are reproducibly observed in NR1- and NR2-induced infiltrates (I), whereas IL-4 positive cells were frequently found in response to NR2 peptide (J). Panels (A), (B), and (C) show paraffin-embedded sections stained with haematoxylin and eosin; magnification, ×400. Panel (D) shows cryo-sections counter-stained with Shandon haematoxylin; magnification ×200. Panel (H) illustrates direct immunostaining, followed by APAAP on a paraffin embedded section; magnification, ×400. In panels (E), (F), (G), (I), and (J), samples were subjected to direct immunostaining, followed by APAAP on frozen sections; magnification ×400 (E, H, I), or ×600 (F, G, J). Sections were counter-stained with Shandon haematoxylin.

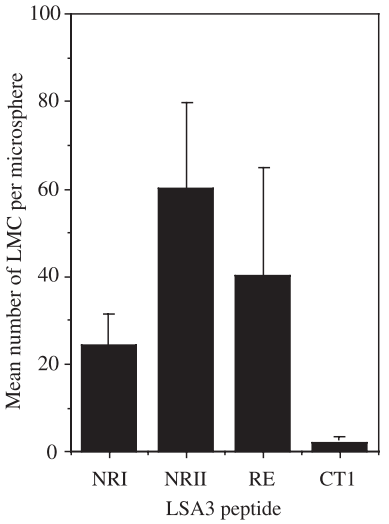


Fig. 2. Mean number (± S.D.) of lymphomonocytic cells recruited by intra-portally delivered LSA3-peptides coated microspheres at 48 h in the liver of LSA-3-729-S-immunised C3H/HeJ mice, using haematoxylin and eosin-stained 5-μm sections. Leukocytes present in 50 granulomas per mouse liver were counted for each peptide. Representative results obtained for three mice are shown for each peptide.

delivery of peptide-coated microparticles revealed at 48 h post-injection that cells surrounding microparticles were mainly composed of $CD3^+$ T lymphocytes and macrophages whatever the peptide delivered. This is reminiscent of the fact that these peptides were previously shown to define strong T cell-epitopes (BenMohamed et al., 1997), particularly NR2, and indeed the highest scores were reproducibly obtained in granulomas resulting from the delivery of NR2-coated microparticles (Fig. 1E). With each peptide, T lymphocytes proved to be of both $CD4^+$ (70 to 80%) and $CD8^+$ (20 to 30%) subsets (Fig. 1F and G). However, their local distribution differed: $CD4^+$ T lymphocytes were frequently seen closer to the microparticles than $CD8^+$ T cells. Macrophages, however, were tightly associated with the microparticles, occasionally covering them entirely, possibly reflecting their phagocytic properties (Fig. 1H).

3.6. Cells infiltrating the granuloma have features of activated cells

The presence of immunocompetent cells around peptide-coated microparticles led us to investigate whether such a peptide-induced microcellular environment had the capacity for in situ antigen presentation and lymphocyte activation. We were particularly interested in studying the ability of cells to produce molecular effectors, such as IFN-γ or IL-4, which provides information on the balance between Th1 and Th2 types of local response. Due to their lability, these cytokines could only be detected in frozen liver sections. We could detect both IFN-γ- and IL-4-producing cells in most granulomas. Cells secreting IFN-γ were reproducibly observed in NR1- and NR2-induced infiltrates (Fig. 1I). Although IL-4 positive cells were also found in both NR1- and NR2-infiltrates, a higher proportion was consistently observed in NR2-induced infiltrates (Fig. 1J). These observations are consistent with the presence of a Th2 rather than a Th1 pattern of effectors in NR2-infiltrates. Interestingly, IL-4-secreting cells were in close vicinity to the microparticles, whereas INF-γ-positive cells were more distant.

4. Discussion

We describe a new method based on the intrahepatic delivery of synthetic peptides coated on polystyrene microparticles into immunised mice as one means of defining more precisely the local cellular immune responses to epitopic peptides.

Besides numerous in vitro studies, in which relevance to in vivo events is debatable, few studies have addressed local immunological events directed to liver stages. Increasing evidence has accumulated over recent years suggesting that cells can migrate very rapidly to various compartments, and that their distribution is far from random (Belkaid et al., 1994). Peripheral immune responses, which are the only ones usually measured, can provide only indirect indications about the local behaviour of effector cells in vivo. Our method permits the rapid screening of new peptides, derived from vaccine candidates, for their ability to initiate lymphocyte recruitment in the liver. It also provides information on the fine epitope specificity of cells recruited locally from polyclonal immune effectors, the intensity of the local cell response, the nature of cells recruited, and finally their phenotype.

By immunohistochemical analysis we could identify numerous macrophages together with $CD3^+$ T lymphocytes of both $CD4^+$ and $CD8^+$ subsets, which have been shown by others to have an important role in protection against the liver forms of *Plasmodium* (Druilhe et al., 1998). It is noteworthy that similar infiltrates of mononuclear cells were observed around schizonts in immunised and protected mice except that $CD4^+$ cells were absent (Khan and Vanderberg, 1992). The fine distribution of T cells differed depending on the sub-type, $CD4^+$ T cells being more numerous and in closer contact to the microparticles than $CD8^+$ T cells. However, the most representative cells of the granuloma were the macrophages which adhered to the microparticles. Although cells of the monocyte cell line were obviously needed to present the antigen to lymphocytes, in this study, the large numbers of macrophages were more likely recruited as a second line of defence at the inflammatory site (Fig. 1H). Thus, it was likely that some peptide molecules broke free from the coated microparticles and were taken up by antigen-presenting cells, promoting inflammatory cascades.

Our analysis revealed the presence of cells with an activated phenotype based upon cytokine secretion. IFN-γ is the most potent cytokine acting against liver schizonts. The cytokine can act directly on the infected hepatocytes by activation of the inducible nitric oxide synthase (iNOS) produced by the target cell itself, or by Küpffer cells. At the same time, it can indirectly mediate the recruitment of other lymphocytes into typical class II-restricted DTH reactions known to be a potent activator of macrophages by itself (Pace et al., 1983a,b; Buchmeier and Schreiber, 1985). Immunostaining for cytokines also revealed the presence of cells able to produce IL4, suggesting a local mixed Th1 and Th2 pattern of response to antigenic stimulation. However, the balance between these two types appeared to depend on the chosen peptide. Whereas the number of cells producing IFN-γ and IL-4 was similar around NR1-coated microparticles, IL-4+ cells were more numerous than IFN-γ+ cells around NR2-coated microparticles.

We also investigated the parallelism between local and peripheral T-cell responses. Whereas the peptide NR2 was the most efficient at initiating lymphomonocytic (LMC) recruitment after intra-portal delivery, spleen cells showed only a weak proliferative response to this peptide (Table 2). The discrepancy is even more striking for peptide RE which induced a very significant local lymphocyte recruitment, but no peripheral proliferative responses were detected against this peptide. With respect to peripheral effectors, peptide NR2 was the most antigenic in terms of IFN-γ secretion since the highest levels of the cytokine were found in the supernatants of spleen cell cultures upon in vitro restimulation with this peptide, whereas only limited amounts were detected in response to the peptide NR1. In contrast, NR1- and NR2-coated microparticles showed a similar ability to recruit IFN-γ-secreting cells locally.

In conclusion, our studies have shown that LSA3-derived peptides differ in their ability to initiate lymphomonocytic cell recruitment in the liver of immune mice. We found that these differences do not directly correlate with the peripheral responses to the same peptides. In order to rationalise vaccine development, this study suggests that it is of importance to extend immunological investigations to local responses and not only to classical peripheral cell analysis. The method described using LSA3 has obvious applications to the many antigens expressed by plasmodial liver stages and more generally to molecules derived from other intra-hepatic pathogens, such as hepatotropic viruses, and *Listeria*. Both in malaria and hepatitis, cellular mechanisms are likely to play a crucial role in defence against the infected hepatocyte (Chisari and Ferrari, 1995; Pasquetto et al., 2000). Cells secreting IFN-γ of both CD4 and CD8 subsets have been implicated in defence. The method described here may help to differentiate at the local in-situ level between various antigen-delivery systems in which one can induce an optimal recruitment of cells with an effective phenotype.

Acknowledgements

We thank Genevieve Milon for helpful discussion and critical review of the manuscript. This work was supported by Grants from the European Commission no. 98 0387 and the PAL+ commitee of the Ministere de la Recherche.

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