Immunology 1991 **73** 88–94 ADONIS 0019280591001161

Differential presentation of hepatitis B S-preS(2) particles and peptides by macrophages and B-cell like antigen-presenting cells

J.-P. Y. SCHEERLINCK,* G. BURSSENS,*† L. BRYS,* A. MICHEL,‡ P. HAUSER† & P. DE BAETSELIER*

**Institute of Molecular Biology, Vrije Universiteit Brussel, St-Genesius-Rode, †Biological division, Smith-Kline Biologicals, Rixensart and ‡Laboratoire de Chimie Biologique, Université de l'Etat à Mons, Belgium*

Accepted for publication 31 January 1991

SUMMARY

Different cell types, including dendritic cells, macrophages and Ia$^+$ B cells, have been described to present soluble antigen (Ag) to T-cell hybridomas. However, it is still not clear whether these different cell types can act as antigen-presenting cells (APC) for complex and insoluble Ag such as viral particles. Using yeast recombinant hepatitis B S-preS(2)-containing particles, T-cell hybridomas were generated and used as a tool to study processing and presentation of antigen. Different types of APC were compared in regard to their capacity to process and present the protein–lipid composed S-preS(2) particles and the thereof derived T-cell epitope containing peptides by different types of APC. While a S-preS(2)-derived T-cell epitope containing peptide, which does not require processing, could be presented both by macrophage and B-cell like APC, the presentation of S-preS(2) particles required the presence of macrophages. The fact that B-cell like APC and macrophages behave differently with regard to the presentation of S-preS(2) particles suggests that the uptake and/or processing of this type of Ag by B-cell like APC and macrophages is different.

INTRODUCTION

The surface Ag of the hepatitis B virus (HBsAg) is encoded by a gene containing three different origins of translation which are in the same phase. By this genetic arrangement three related proteins are formed, namely, the S-preS(2)–preS(1) protein (large protein) starting at the first, the S-preS(2) protein (middle protein) at the second and the S protein (small protein) at the third origin of translation.[1]

A yeast-derived S-preS(2) protein[2] has been used in this study. These S-preS(2) proteins form in association with yeast lipids, very stable particles of 20–25 nm which resemble the 22-nm spherical particles found in the serum of infected patients. In humans, the anti-HBsAg antibody response can lead to the control of infection. In mice, the humoral response against HBsAg is controlled by the major histocompatibility complex (MHC) class II genes.[3,4] Both the preS(1) and the preS(2) parts have been shown to provide additional T- and B-cell epitopes which circumvent the non-responsiveness to the S protein.[5,6] Therefore, the role of T-helper epitopes and the way they are generated and presented could be important for the understanding of the immune response against hepatitis B virus.

Both macrophages and B cells have been described to process and present soluble Ag to primed T cells and T cell hybridomas upon which these T cells produce lymphokines. While the aim of the presentation by macrophages and other Ag-presenting cells is to activate T cells, the presentation by B cells would result in an Ag-specific T–B-cell collaboration. Indeed, B cells were shown to be able to present Ag which is recognized by their surface immunoglobulin, at a concentration 1000 times lower compared to a non-specific Ag. Consequently, at low Ag concentrations, only the Ag recognized specifically by the B-cell is presented, resulting in an Ag-specific T–B collaboration.[7,8] This model implies that the same T-cell receptor must recognize the Ag presented either on the macrophage or on the B cell and hence the Ag has to be presented in the same way by these APC.

In this report we describe that while a T-cell epitope-containing peptide could be presented as efficiently by Ia$^+$ B cells and macrophages, only the latter were able to present total S-preS(2) particles. This might suggest that macrophages and B cells differ in the way they take up and/or process S-preS(2) particles.

Abbreviations: HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; LFA-1, lymphocyte function-associated antigen-1; Ag, antigen; APC, antigen-presenting cells; PEC, peritoneal exudate cell; mAb, monoclonal antibody; FCS, foetal calf serum; IL-2, interleukin 2.

Correspondence: J.-P. Y. Scheerlinck, Dept. of General Biology, Institute of Molecular Biology, Vrije Universiteit Brussel, Paardenstraat 65, B-1640 Sint-Genesius-Rode, Belgium.

MATERIALS AND METHODS

Mice and antigens

Female BALB/c mice, 6–10 weeks old, were purchased from the Animal Breading Centre of the SCK, Mol, Belgium. Yeast-

derived recombinant hepatitis B S-preS(2)- and S-containing particles were produced by Smith-Kline Biologicals, Rixensart, Belgium.[2] The sequence of the S-preS(2) is highly homologous to the sequence described by Valenzuela *et al.*[9] Only four mismatch were detected, i.e. 131 A instead of T, 141 F instead of L, 164 S instead of I, 291 N instead of S. The S2b peptide (156–170: NIASHISSSSARTGD) was synthesized by the Merifield solid-phase method and were subjected to HPLC on a C18 reverse-phase column.

Cell lines

Mice were injected intrafootpad with 2 μg S-preS(2) emulsified in complete Freund's adjuvant (CFA). After 1 week the popliteal lymph node cells were restimulated *in vitro* with 10 μg/ml S-preS(2) particles. After 3 days the living cells were recovered by centrifugation on a discontinuous Ficoll paque gradient (Pharmacia, Uppsala, Sweden) at 550 ***g*** for 20 min. These cells were fused with the HAT-sensitive BW5147 T lymphoma at a ratio of 10 lymph node cells to 1 T lymphoma cell using polyethylene glycol 4000 (Merck, Damstadt, Germany) as described previously.[10] The clones were selected in HAT-containing complete medium [i.e. RPMI-1640 (Gibco, Grand Island, NY) supplemented with 0·30 mg/ml L-glutamine (Flow, McLean, VA) 100 U/ml penicillin, 0·1 mg/ml streptomycin (Gibco), 10% heat-inactivated foetal calf serum (FCS) (Gibco)] supplemented with HAT, 5 10^{-5} M 2-mercaptoethanol (2-ME) and an extra 10% FCS. Later, the T-cell hybridomas were cultured in complete medium supplemented with 2-ME. The B-cell line M12.4[11] and the hybridoma TA3[12] were maintained in complete medium.

Antibodies

Monoclonal antibodies (mAb) recognizing I-A^d (MK-D6),[13] I-$E^{k/d}$ (17-3-3S)[14] and I-A^k (10-3.6.2)[15] were purchased from the American Type Culture Collection (Rockville, MD). S4B6.2 mAb,[16] specific for mouse IL-2, were a kind gift of Dr T. R. Mosmann, from the DNAX Research Institute of Molecular and Cellular Biology, U.S.A. The mAb M17444,[17] specific for the lymphocyte function-associated antigen-1 (LFA-1), was provided by Dr E. Roos from The Netherlands Cancer Institute, The Netherlands.

Antigen presenting cells

Syngeneic spleen cells were used as a source of APC. Serum adherent spleen cells, enriched for macrophages, were prepared by 4-hr adherence on bacterial culture dishes coated overnight with FCS.[18] The non-adherent fraction was further depleted of macrophages by an extra 3 hr adherence on culture dishes (3003; Falcon, Oxnard, CA). The adherent cells were recovered by incubating the bacterial culture dishes for 30 min with 0·2% EDTA in phosphate-buffered saline (PBS) at 4°. Subsequently cells were detached by vigorous pipetting.

Resident peritoneal exudate cells (PEC) were recovered by washing the peritoneal cavity with 10 ml cold PBS containing 5 U/ml heparin (Organon, Oss, The Netherlands) and 4·5 mg/ml D-glucose. Thio-PEC and Con A-PEC were obtained by injecting mice, i.p., with 3 ml of thioglycolate broth (Bio Mérieux, 69260 Charbonnière-les-bains, France) and 10 μg of concanavalin A (Con A; Sigma, St Louis, MO), respectively. On Days 5 and 3, respectively, mice were killed and PEC recovered as described previously. When PEC were used as APC, 10 μg/ml indomethacine were used in order to eliminate the inhibitory prostaglandine production by these cells.[19]

Antigen presentation assay

Antigen-presenting cells at the indicated concentration were mixed with two 10^4 T-cell hybridomas in microwell plates in a final volume of 200 μl. An optimal concentration of Ag was added in the appropriate wells [10 μg/ml for the S-preS(2) particles and 50 μg/ml for the S2b peptide]. When indicated 10 μg/ml of indomethacin (Sigma, St. Louis, MO) were added to the Ag presentation assay, to prevent prostaglandin-mediated suppression of the *in vitro* response. The indomethacin was dissolved at a concentration of 10 mg/ml in ethanol and diluted in complete medium containing 2-ME. After 24 hr of incubation, 100 μl were transferred to a new microwell plate and frozen to eliminate remaining cells. 10^4 CTLL cells[20] (a kind gift of Dr C. Uytenhoven, Université Catholique de Louvain, ICP, Brussels, Belgium) were added to the freeze-thawn supernate and after 24 hr, pulsed with [methyl-^{3}H]thymidine (Amersham, Amersham Bucks, U.K.) for 18 hr before harvesting and measuring the incorporated [methyl-^{3}H]thymidine using a scintillation counter. The results are presented as C.P.M. upon subtraction of background proliferation in the absence of Ag.

Prediction of T-cell epitopes

The prediction of amphipathic sequences, which were shown to correlate with T cell epitopes, was carried out using the computer program AMPHI.[21] Using a Turbo Pascal program the S2b peptide, including adjacent amino acids, was screened for the presence of four or five amino acid sequences corresponding to the pattern described by Rothbard & Taylor.[22]

RESULTS

Generation and characterization of hepatitis B S-preS(2)-specific T-cell hybridomas

To produce hepatitis B S-preS(2)-specific T-cell hybridomas, T cells obtained from S-preS(2)-primed lymph node cells (LNC) from BALB/c mice were fused with the HAT-sensitive T lymphoma BW5147. The generated hybrids were tested for their capacity to produce T-cell growth factors for CTLL cells, in the presence of spleen APC and S-preS(2) particles. Three Ag-specific T-cell hybridoma lines were thus identified that grew rapidly in culture without stimulation but produced T-cell growth factors in response to a challenge with specific Ag and APC. These T-cell hybrids were further characterized with regard to their antigenic specificity, the H-2 restriction elements and the T-cell growth factors produced upon stimulation.

One of the T-cell hybridomas, i.e. HB 242, could be stimulated by S particles as well as by S-preS(2) particles, while the T-cell hybrids HB 82 and HB 68 could only be stimulated by S-preS(2) particles, indicating that these two clones were preS(2)-specific (Table 1). To further investigate the antigenic specificity of the two preS(2) specific T-cell hybridomas, synthetic peptides corresponding to predicted amphipathic sequences of preS(2), as defined by the computer program AMPHI[21] were synthesized. The peptide S2b (156–170) corresponding to a sequence in the preS(2) region was found to be antigenic for both HB 68 and 82, confirming the preS(2) specificity of these hybridomas (Table 1). Other peptides derived

Table 1. Characteristics of the T-cell hybridomas

	Antigenic specificity*			% MHC restriction†			% LFA-1‡	
T hybridomas	S-preS(2)	S	S2b	I-A^d	I-E$^{k/d}$	I-A^k	S2b	S-preS(2)
HB 68	54±3	0±1	107±2	95	0	0	39	97
HB 82	64±4	0±1	87±1	92	0	0	43	91
HB 242	13±2	5±1	0±1	48	5	4		

* The antigenic specificity was measured by the capacity of the different antigens to stimulate the T-cell hybridomas in the presence of spleen cell APC. All experiments were performed in triplicate and the mean of triplicates ± standard deviation are shown. CTLL proliferation was measured following addition of supernates collected from co-cultures of APC and T-cell hybridomas, with and without antigen. The results are expressed in Δ C.P.M. × 10^3 of [^{3}H]thymidine incorporation. Δ C.P.M. = C.P.M. with antigen − background C.P.M. without antigen. In all cases the background was lower then 2 × 10^3 C.P.M.

† The MHC restriction was assessed by adding anti-MHC class II monoclonal antibodies to the co-cultures. As a control a monoclonal antibody against an irrelevant haplotype (I-A^k) was used. The results are expressed as percentage inhibition if the response without addition of antibodies.

‡ The dependence of the T-cell stimulation on the accessory molecule LFA-1 was assessed by adding anti-LFA-1 monoclonal antibodies to the antigen presentation assay in the presence of S2b or S-preS(2) as antigen. The results are expressed as a percentage inhibition of the Ag-specific production of IL-2 upon addition of the antibodies.

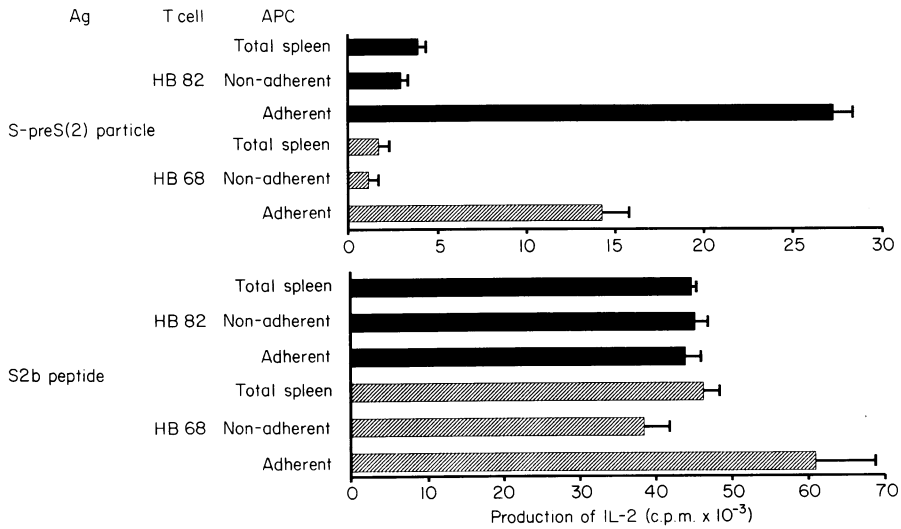

Figure 1. Presentation of S-preS(2) particles and S2b peptides by spleen cell subpopulations. Spleen cells were fractionated in FCS-adherent cells and in FCS and plastic non-adherent cells. 2 × 10^4 APC and 2 × 10^4 T-cell hybridomas were used in each well. The results are expressed as indicated in Table 1. All experiments were performed in triplicates and standard deviations are shown.

from the S (i.e. 193–207, 197–215, 289–304, 330–345, 374–389), preS(2) (i.e. 120–145) and the overlapping region between S and preS(2) (i.e. 168–180, 172–184) parts of the S-preS(2) particles, were all found to elicit no significant response from the T-cell hybridomas (data not shown).

Using monoclonal antibodies against class II MHC molecules, the Ia restriction of the T-cell hybridomas was investigated (Table 1). The monoclonal antibody, MK-D6, recognizing I-A^d could inhibit the presentation of S-preS(2) particles as well as S2b peptides to both preS(2)-specific T-cell hybridomas. The I-E$^{k/d}$-specific monoclonal antibody, 17-3-3S, exhibited a marginal inhibition of the Ag-specific response which was also observed when an irrelevant mAb (i.e. 10–3.6.2 specific for I-A^k) was used as a negative control. These results indicate that the presentation of S-preS(2) particles and S2b peptides to the T-cell hybridomas is I-A^d restricted. This is also the case for the presentation of S-preS(2) to the S-specific T-cell hybridoma HB 242.

The accessory molecule LFA-1 was described to be involved in T cell–APC interactions by enhancing cell–cell contact. However, this adhesion molecule was found not to be involved in the activation of the same T-cell hybridomas by Ag presented by Ia-transfected L cells, indicating that LFA-1 is not essential for Ag-specific T-cell activation.[23] To investigate the role of this

Table 2. Presentation of S-preS(2) particles by peritoneal exudate cells

T hybridomas	Spleen	Macrophage subset*		
		Resident PEC	Thio-PEC	Con A-PEC
HB 68	17±3	31±1	26±1	48±3
HB 82	38±9	50±1	59±1	105±7

* Macrophages were obtained from *in vivo*-stimulated peritoneal exudate cells, and used as APC at a concentration of 2×10^4 cells/well. Spleen cells, at a concentration of 2×10^5 cells/well, were added as a positive control for this experiment. All experiments were performed in triplicate and the mean of triplicates ± standard deviation are shown.

CTLL proliferation was measured following addition of supernates collected from co-cultures of APC and T-cell hybridomas, with and without antigen. The results are expressed in Δ C.P.M. $\times 10^3$ of [^{3}H]thymidine incorporation. Δ C.P.M. = C.P.M. with antigen − background C.P.M. without antigen. In all cases the background was lower then 2×10^3 C.P.M. 10 μg/ml indomethacin were added, in all wells, to avoid suppression by prostaglandins produced by the PEC.

molecule in the Ag-specific response of the hepatitis B preS(2)-specific T-cell hybridomas, the monoclonal antibody, M17444, specific for LFA-1 was added during Ag presentation. The results shown in Table 1 indicate that the Ag-specific response of the T-cell hybridomas was drastically suppressed by this antibody when the cells were stimulated by S-preS(2), while a less pronounced but significant suppression was recorded upon stimulation with S2b.

The proliferation of CTLL cells can be mediated by IL-2 as well as IL-4.[24,25] Using a monoclonal antibody, S4B6.2, directed against mouse IL-2[16] we have investigated which of these lymphokines was produced by the activated T-cell hybridomas. This antibody could inhibit CTLL proliferation induced by supernate derived from S2b- and S-preS(2)-stimulated T-cell hybridomas for at least 85%, indicating that both S-preS(2)- and S2b-specific stimulation of the T-cell hybridomas leads to secretion of IL-2.

Presentation of S-preS(2) particles by spleen cell subpopulations

From Table 1 it is clear that spleen APC present more efficiently the S2b peptide than the S-preS(2) particles. This result indicates that the presentation of these two antigenic molecules might be subjected to different mechanisms. In fact, in contrast to the S2b peptide, the S-preS(2) particle could not be presented by formaldehyde-fixed APC (data not shown). This suggests that while the S-preS(2) particle needs active processing to be presented to the T-cell hybrids, the S2b peptide does not require a processing step for optimal presentation. In order to investigate possible differences in the uptake, processing and presentation of S-preS(2) particles versus S2b peptides, by various cell types, we have compared different spleen cell subpopulations for their ability to present these two antigens to the T-cell hybrids. Therefore, the spleen cells were subfractionated in serum adherent macrophage-enriched cells[18] and non-adherent macrophage-depleted cells. As shown in Fig 1, both fractions could present S2b peptides to the same extent, while the adherent cell fraction could present S-preS(2) particles more effectively than the total spleen population or the non-adherent fraction. Since macrophage depletion from spleen cells by FCS and plastic adherence leads to reduction of S-preS(2) particle presentation, it is likely that the presentation of S-preS(2) particles is macrophage dependent. In contrast, the presentation of the S2b peptide is only moderately affected by the removal of the adherent cells, indicating that the presentation of S2b does not require the presence of macrophages.

In order to confirm the role of macrophages in the presentation of S-preS(2) particles, several sources of PEC were compared in the same experiment. The results shown in Table 2 indicate that resident, as well as *in vivo*-induced , thioglycolate

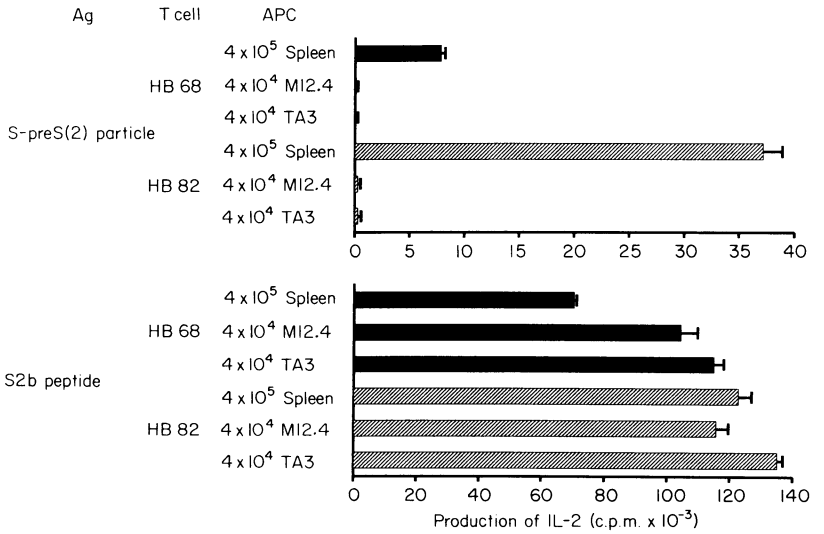

Figure 2. Presentation of S-preS(2) particles and S2b peptides by Ia$^+$ B-cell lines. An optimal concentration of B-cell like APC for the presentation of S2b was used. As a positive control an optimal concentration of spleen cells was used. The results are expressed as indicated in Table 1. All experiments were performed in triplicates and standard deviations are shown.

and Con A-PEC are able to present S-preS(2) particles to the hepatitis B-specific T-cell hybridomas.

Ia$^+$ B-cell lines are unable to present S-preS(2) particles

In order to confirm that S-preS(2) particles cannot be presented by B-cell-like APC, Ag-presenting Ia$^+$ B-cell tumour lines were tested. The M12.4 B lymphoma [11] and the TA3 hybridoma,[26] a fusion product between M12.4 cells and LPS-stimulated spleen cells, have been described as potent APC for several soluble Ag.[12,27] These cell lines have been compared for their capacity to process and present the S2b peptide and the S-preS(2) particle. As shown in Fig. 2 both cell lines were unable to activate the T-cell hybridomas in the presence of S-preS(2) particles. However, when the S2b peptide was used as a source of Ag the two preS(2)-specific T-cell hybridomas could be activated by these B-cell lines even more efficiently than by spleen cell APC. Furthermore, the presentation of S2b peptide by these B-cell lines could be inhibited by monoclonal antibodies directed to IA^d indicating that these B-cell lines express functional I-A^d molecules (data not shown).

DISCUSSION

Ag-specific T-cell hybridomas were generated, in BALB/c mice, against yeast-derived recombinant hepatitis B S-preS(2) particles. These T-cell hybridomas were shown to produce IL-2 upon antigen-specific stimulation. Since the secretion of IL-2 and IL-4 is claimed to be mutually exclusive,[28] this result suggests that the T-cell hybridomas should be classified as belonging to the Th 1 cells according to the classification of Mosmann *et al.*[16] Moreover, the presence of the T200 antigen on the cell surface of the hybridomas supports this view (data not shown). Two of the T-cell hybridomas recognized the preS(2) part of this Ag and were further characterized. Using synthetic peptides, the recognized T-cell epitope was found to be located within the S2b peptide (156–170). This peptide contains in a region with an amphipathic character, as predicted by the program AMPHI,[21] but does not contain a T-cell epitope-related pattern as described by Rothbard & Taylor.[22] Milich *et al.*[29] described an equivalent T-cell epitope (156–170) in a hepatitis B variant of the adw2 serotype, using mice of the H-2^d haplotype. This epitope is not exactly the same as the S2b epitope since ILE 164 is replaced by SER 164. In humans, the preS(2) part of the HBsAg does not seem to be immunodominant as compared to the S and preS(1) regions.[30] Within the preS(2), the HBsAg region 120–145 was shown to contain a major T-cell epitope.[31] However, in the present work, in mice the major preS(2) T-cell epitope was found to be located at the COO$^-$ terminal side of the preS(2) region. Furthermore, none of the generated T-cell hybridomas was found to be reactive with the peptide corresponding to the 120–145 region of the used hepatitis B variant (data not shown).

The comparison of the Ag presentation capacity of different cell populations, for both S-preS(2) particles and S2b peptides, revealed that the optimal presentation of S-preS(2) particles required a macrophage type of APC, while the S2b peptide could also be presented by a B-cell type of APC.

In several experimental systems different mechanisms were proposed to explain the differential behaviour of B cells and macrophages with regard to T-cell activation, i.e. (i) differential expression of accessory molecules[32] or Mls Ag,[33] (ii) qualitative differences in processing [32] or presentation,[34–36] and (iii) quantitative differences in Ag uptake and processing.[37]

Since the S2b peptide could be presented by fixed APC and hence did not require extensive processing, it is possible that B cells and macrophages take up and/or process the S-preS(2) particles differentially in a quantitative and/or qualitative way. Qualitative differences in Ag processing might lead to the generation of different peptides by different types of APC. As a consequence these differences were expected to be related to particular features of the T-cell epitopes. However, the tested B-cell lines were unable to present S-preS(2) particles to any of the S-preS(2)-specific T-cell hybridomas, including not only the two preS(2)-specific hybridoma, HB 68 and HB 82, but also the S-specific T-cell hybridoma HB 242. Hence, it is unlikely that the lack of presentation of S-preS(2) by B-cell lines would depend only on qualitative differences in processing or presentation.

In contrast, quantitative differences such as defective uptake and/or processing of S-preS(2) particles are more likely to account for the inability of B-cell like APC to present such particles to S-preS(2)-specific T-cell hybridomas. Hereby it should be emphasized that, in contrast to other experimental systems where the processing of Ag by B-cell tumours and macrophages was described to be similar (i.e. ovalbumin),[38] the Ag used in this study was a lipid-associated protein. Liposome-like particles may require more specialized cells for an efficient uptake and degradation, as suggested by the studies of Dal Monte & Szoka.[37] Although the S-preS(2) particles (20–25 nm) are much smaller then the liposome used by these investigators (312 nm) it is possible that the S-preS(2) particles mimic to some extent a stable liposome that can not be taken up and subsequently presented by B-cell like APC.

The results described here do not imply that B cells are completely incompetent APC for S-preS(2) particles. Indeed, the actual presentation experiments were performed with either naive B-cells or S-preS(2) non-specific B-cell lines but not with antigen-specific B cells. In this context it is appropriate to refer to the observations of Milich *et al.*[39] showing that mice primed with the hepatitis B core Ag (HBcAg) or a T-cell epitope containing core peptide, gave rise to a high Ab production against HBcAg as well as HBsAg, when boosted with the total hepatitis B virus. However, when boosted with a mixture of HBcAg and HBsAg no secondary immune response against HBsAg could be detected, indicating that HBcAg-specific T cells can provide bystander help to HBsAg-specific B cells provided both antigens are linked. Since in intact viral particles HBsAg and HBcAg are not covalently linked but associated through a liposome-like structure, one has to assume that the HBsAg-specific B cells are able to take up and process intact particles, containing both the HBsAg (for B-cell specificity) and the HBcAg (for T-cell specificity). If one assumes that the uptake of S-preS(2) particles by B cells limits the ability of these APC to present this Ag, it is possible that Ag-specific B cells would circumvent this inability by increasing the Ag uptake using their surface Ig.

ACKNOWLEDGMENTS

J.-P. Y. Scheerlinck is a research assistant of the Nationaal Fonds voor Wetenschappelijk Onderzoek (N.F.W.O.). P. De Baetselier is a research associate of the N.F.W.O.

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