ELSEVIER

International Journal of Pharmaceutics 155 (1997) 19–25

international journal of pharmaceutics

Antibody responses in rabbits after single immunization with viral glycoprotein-loaded microspheres or ISCOMs

Jaroslav Hampl [a,*], Dagmar Pokorova [a], Jaroslav Franz [a], Milan Dittrich [b], Jan Štěpánek [a]

[a] *Veterinary Research Institute, 621 32 Brno, Czech Republic*
[b] *Faculty of Pharmacy, Charles University, 500 05 Hradec Kralové, Czech Republic*

Received 22 January 1997; accepted 9 May 1997

Abstract

Antigenic and adjuvant activities of microspheres and ISCOMs (immunity stimulating complexes) with incorporated envelope glycoproteins of bovine herpesvirus-1 (BHV-1) were tested in long-term (31-week) experiments in rabbits. Similar antibody responses were recorded after a single intradermal immunization with BHV-1 glycoproteins incorporated into ISCOM nanoparticles or microspheres prepared from linear co-polymers (50/50) of DL-lactic and glycolic acids. Stronger, longer and markedly biphasic antibody responses were observed in rabbits immunized with BHV-1 M-DL-LA microspheres prepared from branched oligoesters of DL-lactic acid and mannitol. Indirect evidence of the incorporation of the viral envelope proteins into any type of microspheres or ISCOMs was obtained by immunoblotting. A set of precipitation lines was detected in rabbit blood sera collected 7 weeks after the immunization with microsphere- or ISCOM-entrapped glycoproteins. A similar, but more numerous, set was found in the control serum collected from cattle after natural infection by BHV-1. © 1997 Elsevier Science B.V.

Keywords: Microspheres; Linear and branched polyester structures; ISCOMs; Bovine herpesvirus-1; Antigen formulation; Adjuvant activity

1. Introduction

The strategy of the development of vaccines, that are still necessary for the control of infectious diseases, has been changing rapidly during the past 10–15 years. Advances in several branches of science, such as immunology and genetic engineering, have allowed the identification and

* Tel. +24 5 41321241; fax. +42 5 41211229.

PII S0378-5173(97)00144-0

preparation by various techniques of a broad range of individual antigens of pathogenic agents capable of inducing immune responses. Thus a basis has been established for the development of subunit vaccines which, unlike the conventional types, contain accurately defined antigens. At the same time, it is necessary to develop new ways of presentation of antigens to the immune system, owing to the low antigenicity of the subunits. Adjuvant formulations seem to be more effective for this purpose than the conventional adjuvants. The principle of the formulations consists of the co-incorporation of soluble subunit antigens and adjuvants into common multimolecular structures. Typical examples of such structures are immunity stimulating complexes—ISCOMs (Morein et al., 1984) or liposomes (Gregoriadis, 1990). This group also includes biologically compatible and degradable microspheres (msp) prepared from synthetic polymers, that have shown good antigen-carrier properties in recent studies (Eldridge et al., 1991; Cahill et al., 1995). Adjuvant effects of msp, particularly those from lactic and glycolic acid polymers, equal to those of the conventional adjuvants, such as Freund's adjuvant or aluminium hydroxide (O'Hagan et al., 1991). The selection of suitable polymers and technology of preparation of msp allows the establishment of a controlled-release delivery system that offers a prospective alternative of adjuvant formulation, among other applications also for the development of subunit vaccines (Cleland et al., 1994).

Our recent comparative studies of adjuvant activities of msp prepared from linear or branched lactic or glycolic acid oligoesters with incorporated BSA, and their degradation in vitro in PBS and blood serum (Hampl et al., 1996), have demonstrated adverse effects of the components of blood serum on the degradability. This paper describes experiments with the two most readily degraded types of msp prepared from a branched (M-DL-LA) and a linear (DL-PLGA 50/50) oligoesters, respectively, using envelope glycoproteins of bovine herpesvirus-1 as a model antigen.

2. Experimental

2.1. Materials

The following chemicals were used in the experiments: polyvinylalcohol (PVA) M_r 85–140 kDa, 86–89%, hydrolyzed (Aldrich, USA); dichloromethane (DCM); DL-PLGA with the co-polymer ratio of 50/50 (lactide/glycolide %) and M_r 50–75 kDa; gelatin Type A (300 Bloom); octylglycoside; cholesterol; phosphatidylcholine; 3,3′,5,5′-tetramethylbenzidine; horseradish peroxidase; sodium dodecyl sulfate (all by Sigma, USA); Quil A (Superfos, Biosector, Denmark); lactalbumin hydrolysate (Difco, USA). The terpolymer M-DL-LA, M_r 5.3 kDa, was prepared in our laboratory using the polycondensation method (Hampl et al., 1996).

2.2. Isolation of BHV-1 glycoproteins

The procedure was described in detail elsewhere (Franz et al., 1996). Briefly: The virus ($10^{6.0}$TKID$_{50}$ per 0.05 ml) was propagated in a monolayer of MDBK cells. After ultracentrifugation of the clear supernatant of infected cells at 27 000 r.p.m. in the rotor Sw 28 (Beckman Instrument, USA) for 1 h, the sedimented virus was resuspended and subsequently purified in a 30/50 (w/v) gradient of potassium tartrate. The virus-containing fractions were pooled and envelope glycoproteins were released from the purified virus by incubation in 2% octylglucoside for 1.5 h and separated by centrifugation at 40 000 r.p.m. in the rotor Sw 55 for 1 h. The resulting supernatant containing envelope glycoproteins was then used in the preparation of microspheres and ISCOMs.

2.3. Preparation of microspheres

Msp were prepared from the two types of polyesters by the solvent evaporation emulsion technique (w/o/w) as described by Jeffery et al. (1993). The procedure was as follows: 100 mg polyester were dissolved in 3.0 ml dichloromethane, and the viral glycoprotein, along with the identical ^{125}I-labelled tracer, in 0.85

ml of 0.2% gelatin. The two solutions were cooled to 4°C pooled in the homogenizer Ultra-Turrax and dispersed at 20 000 r.p.m. for 1 min. The emulsion was immediately transferred into 5 ml 2% PVA containing 0.5% Poloxamer 188, cooled again to 4°C and dispersed at 20 000 r.p.m. for 1 min. The resulting emulsion was stirred at 300 r.p.m. and 20°C for 16 h. Then the suspension of msp was washed twice with 25 ml distilled water containing 0.5% Poloxamer 188. Each washing step was followed by centrifugation at 4000 r.p.m. The rate of entrapment of viral glycoprotein into msp was calculated from the radioactivity of the non-entrapped tracer in the supernatant. The separated msp were resuspended in 1% Poloxamer 188, freeze-dried and kept in sealed ampoules at 4°C.

2.4. Preparation of ISCOMs

Viral glycoproteins were mixed with cholesterol and phosphatidylcholine at the ratio of 2.5:1:1 and Quil A was added to reach the final concentration of 0.1%. Octylglucoside was removed by extensive dialysis against 0.1 M ammonium acetate. The dialysate was centrifuged through a layer of 10% saccharose at 40 000 r.p.m., rotor Sw 55, for 4 h. The ISCOM-containing sediment was resuspended in PBS, pH 7.8 and examined by electron microscopy. The rate of glycoprotein incorporation was determined using the ^{125}I tracer.

2.5. Morphology and size of microspheres and ISCOMs

The preparation and sizes of ISCOMs were monitored using the electron microscope Tesla BS 500. Surface smoothness and morphology of msp were examined by scanning electron microscopy at magnifications 1000 and 20 000 using the microscope Tesla BS 300. The procedure of size distribution analysis of msp is described elsewhere (Hampl et al., 1996).

2.6. Biological experiment

Antigenic and adjuvant properties of M-DL-LA and DL-PLGA msp and ISCOMs with incorporated glycoproteins were tested in three groups of four rabbits.

A single intradermal dose of 0.3 ml PBS, pH 7.2, containing 25 μg of viral glycoprotein in 40.3 and 44.4 mg of M-DL-LA and DL-PLGA, respectively, or ISCOMs, was distributed to six injection sites in the back region.

Blood samples were collected from the ear vein 3 weeks after the inoculation and further samplings continued at 2-week intervals up to week 31. The samples were frozen at -20°C, and tested by ELISA after the sampling period was over.

2.7. Serology

The blood serum samples were tested by indirect ELISA with the viral and the control antigens bound to the solid phase. The viral antigen was prepared from BHV-1 propagated in MDBK cells with a medium supplemented with foetal calf serum. The antigen was concentrated as described above, omitting the centrifugation in the density gradient. The control antigen was prepared in the same way from a non-infected cell culture. The conjugate was obtained by labelling porcine anti-rabbit antibodies with horse-radish peroxidase. Hydrogen peroxide and tetramethylbenzidine were used as the substrate. The blood sera were tested in duplicates at the dilution 1:100. The results are expressed in terms of differences in O.D. (450 nm) between wells containing the viral and the control antigens. The paired t-test and the software Graph Pad Prism Version 2.00 were used to test the significance of differences among individual groups.

2.8. SDS-page and immunoblotting

A modification of the method of Laemli (1970), in polyacrylamide gel under reducing conditions and in the presence of sodium dodecyl sulphate was used. The separated BHV-1 proteins were transferred electrophoretically onto nitrocellulose membrane for immunoblotting (Bio-Rad Laboratories, USA) (Towbin and Gordon, 1984).

After blocking with lactalbumin hydrolysate, the membranes were incubated in rabbit blood

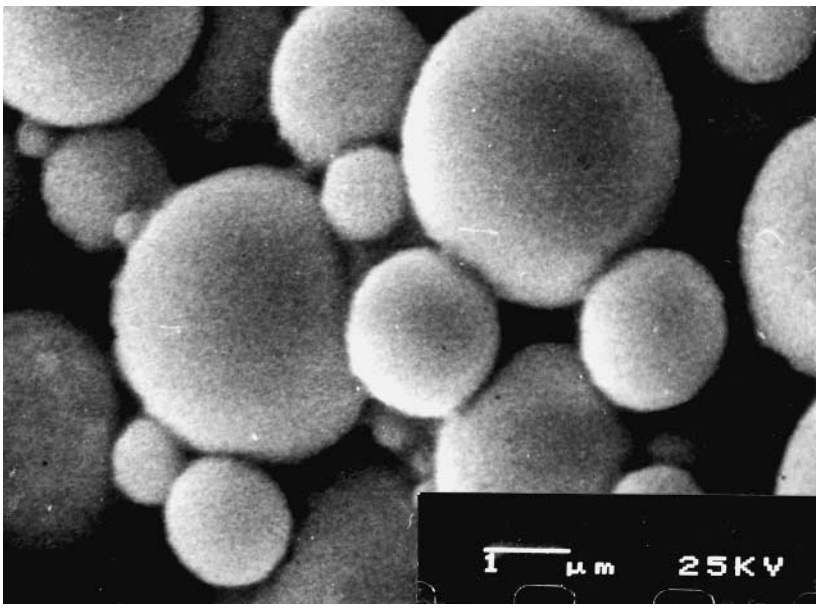

Fig. 1. Scanning electron micrographs of BHV-1 glycoprotein-loaded M-DL-LA microspheres (Surface details, magnification $\times$ 20 000).

sera collected 7 weeks after the immunization with DL-PLGA or M-DL-LA msp or ISCOMs with incorporated BHV-1 glycoproteins. Bovine serum obtained from an animal naturally infected by BHV-1 was used as the control. After binding with horse-radish peroxidase-labelled antibodies to rabbit or bovine immunoglobulins, the specifically reacting antibodies were visualized with diaminobenzidine and hydrogen peroxide as the substrate.

3. Results and discussion

The formation and size of ISCOMs, ranging between 30 and 40 nm, was checked by electron microscopy. Scanning electron microscopy. confirmed that msp prepared by the w/o/w method were free of superficial artefacts. An example of M-DL-LA msp is shown in Fig. 1. The results of size distribution analysis are presented in Table 1.

Antibody responses to BHV-1 glycoproteins incorporated into two types of microspheres or ISCOMs were investigated in a long-term (31 weeks) experiment in rabbits.

As the high antigenicity of BHV-1 ISCOMs was already confirmed in earlier papers (Trudel et al., 1987; Merza et al., 1991; Franz et al., 1996), this vehicle, presenting also the antigen in its corpuscular form, was used as a control in this experiment.

As seen in Fig. 2, all the treated groups responded to the intradermal administration of the antigen with a gradual increase of blood serum antibody levels reaching their maxima around week 7. A small decrease in antibody levels was observed up to week 11 in rabbits treated with BHV-1 ISCOMs. This decrease was followed by a plateau up to week 25 and a marked decrease during the subsequent 4 weeks.

The dynamics of antibody responses in the group immunized with BHV-1 DL-PLGA 50/50 closely resembled those of the control group except for an indistinct second increase after week 13. The decrease from week 25 was similar to that observed in the control group.

The group immunized with BHV-1 M-DL-LA msp differed from the preceding two by a marked second increase in serum antibody levels from week 13 and a shift of the subsequent decrease to

Table 1
Microsphere size distribution (μm)

Vehicle	<1	1–2	2–3	3–4	4–5	>5
DL-PLGA(50/50)	10 (%)	24 (%)	31 (%)	20 (%)	10 (%)	5 (%)
M-DL-LA	16 (%)	29 (%)	46 (%)	8 (%)	1 (%)	0 (%)

week 27. The highest and the lowest titres of blood serum antibodies at the end of the experiment in week 31 were recorded in the groups immunized with DL-PLGA 50/50 msp and ISCOMs, respectively.

The dynamics of antibody response in the group treated with M-DL-LA msp differed significantly from those in the groups treated with DL-PLGA 50/50 msp ($P = 0.025$) or ISCOMs ($P = 0.0003$). No signficant difference was found between the latter two groups ($P = 0.1173$).

Biphasic antibody responses with the first peak in week 3 were found in our earlier experiments in which single doses of M-DL-LA, GA-M-DL-LA, DL-PLGA 50/50 or DL-PLGA 75/25 with incorporated BSA were administered subcutaneously to mice (Hampl et al., 1996). In the present experiment, all three groups of rabbits showed maximum antibody titres as late as in week 7. This delay may have been due to the fact, that the inoculum was administered intradermally, i.e. into tissues with a lower blood flow providing better conditions for the establishment of a depot space for msp, while the presentation of ISCOM-entrapped antigens takes a different course (Watson et al., 1989; Van Binnendijk et al., 1992).

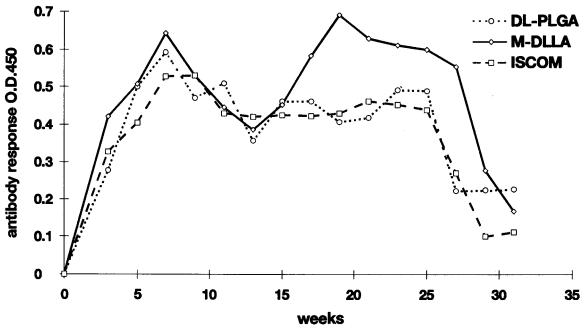

Fig. 2. Antibody responses in rabbits after a single immunization with viral glycoprotein loaded DL-PLGA, M-DL-LA microspheres or ISCOMs.

The persistence of antibody responses after a single shot injection was approximately the same in all three groups. A marked decrease of antibody levels was observed around week 30. Our results are similar to those reported by Alonso et al. (1994) for mice treated with a single subcutaneous dose of msp with entrapped tetanus toxoid in which maximum IgG levels were found between weeks 4 and 8 and their decrease in week 26. Similar results were also obtained by Cleland et al. (1994) after a single dose of PLGA msp with encapsulated MN rpg 120 protein, i.e. a subunit antigen of HIV-1.

Nakaoka et al. (1995) monitored antibody responses in mice treated with a single dose of microspheres with entrapped egg albumin administered by three various routes. Maximum antibody responses were reached 7–14 weeks after the immunization and high titres persisted up to week 22. The authors explained the prolonged period of antibody production as a result of a rise of a memory cell population.

Biphasic immune responses in mice treated with a single subcutaneous dose of microspheres with entrapped tetanus toxoid were reported by Thomasin et al. (1996). The administration of small msp accelerated the onset of the first phase of the immune response, while the administration of larger msp, prepared from polymers with a low degradability, resulted in a marked delay of this onset.

The period of antibody response in animals immunized with ISCOM antigens can be modulated by changing the dose of the antigen (Trudel et al., 1987). Microspheres offer one way more, consisting in the selection of polymers used in their preparation. To accelerate the availability of the antigen to further processing in antigen-presenting cells, we used msp with a very rapid degradability in biological materials (Hampl et al., 1996).

Differences in dynamics of antibody responses of rabbits immunized as model animals with equal doses of antigen incorporated into DL-PLGA or M-DLLA msp became apparent particularly in the second half of the observation period, when O.D. values in the rabbits treated with M-DL-LA msp were higher. This conceptually unambiguous result cannot be regarded as conclusive, however, because only one level of msp treatment was tested. A dose-response design of experiments with BHV-1 incorporated into M-DL-LA or DL-PLGA msp will be sensible only when carried out in target species, i.e. those sensitive to BHV-1.

The preparation of msp by the w/o/w method includes the use of organic solvents that can damage the structure and stability of the entrapped protein or even denature it. Therefore, the inner phase of the emulsion was supplemented with 0.2% gelatin as a protective colloid and stabilizer (Chang and Gupta, 1996). As demonstrated in vitro, the administration of the msp types used in their experiments is accompanied by an enhancement of the initial burst effect which can eventually potentiate the primary antigenic stimulus.

The harmless incorporation of viral glycoproteins into both msp types and ISCOMs without affecting their antigenic structure is evidenced not only by specific antibody responses in ELISA, but also indirectly by results of immunoblotting (Fig. 3). Rabbit antibodies to BHV-1, present in blood serum samples collected in 7 weeks after the immunization, gave rise to a set of lines with a distribution resembling those obtained with blood serum collected from cattle naturally infected by BHV-1, although their number was smaller. The pattern of the lines obtained from blood sera of animals immunized with ISCOMs was somewhat different, particularly as far as their intensity was concerned. This was obviously due to preferential incorporation of amphipathic molecules into the structure of ISCOMs. Of the set of glycoproteins forming the envelope of BHV-1, gI, gIII and gIV with molecular masses 130, 91 and 71 kDa, respectively, are important for the production of protective antibodies (Van Drunnen Little-Van den Hurk and Babiuk, 1986). Although studies of actual protective activity of the produced antibodies were out of the scope of the experiments reported here, the results of immunoblotting are suggestive of the presence of protective antibodies.

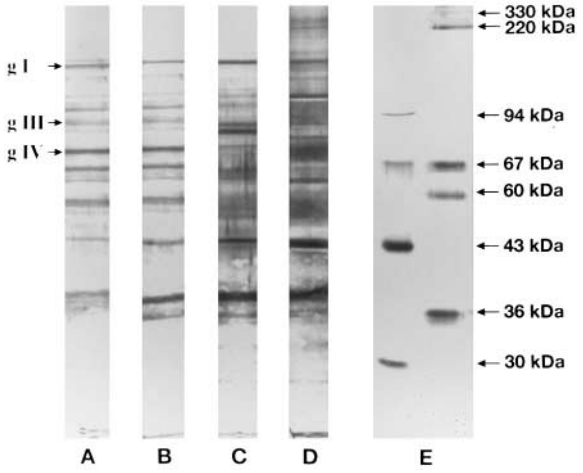

Fig. 3. Immunoblotting with rabbit and bovine antibodies to BHV-1. Rabbits were immunized with viral glycoprotein-loaded PLGA msp (A), M-DLLA msp (B), or ISCOMs (C), bovine serum was collected from an animal naturally infected by BHV-1 (D), molecular weight markers (E).

The demonstration of antibody formation in rabbits immunized with BHV-1 glycoproteins incorporated into M-DL-LA or DL-PLGA microspheres has confirmed that the preparation process, pre-tested only with BSA (Hampl et al., 1996) does not result in a destruction of viral proteins or degradation of their antigenic structure. A similar conclusion can be drawn from comparisons of levels and persistence of antibodies to BHV-1 of the experimental and control groups of rabbits immunized with antigens entrapped into microspheres and ISCOMs, respectively, i.e. vehicles in which marked immunostimulatory activities had been confirmed earlier (Franz et al., 1996). The incorporation of microbial proteins into microspheres, currently tested with clearly defined practical intentions in human medicine (Esparza and Kissel, 1992; Chandrasekhar et al., 1994; Cleland et al., 1994), is obviously a prospective method usable also in veterinary medicine.

Acknowledgements

This work was supported by the Ministry of Agriculture of the Czech Republic (Grant No. 5565)

References

Alonso, M.J., Gupta, R.K., Min, C., Siber, G.R., Langer, R., 1994. Biodegradable microspheres as controlled release tetanus toxoid delivery systems. Vaccine 12, 299–306.

Cahill, E.S., O' Hagan, D.T., Illum, L., Barnard, A., Mills, K.H.G., Redhead, K., 1995. Immune responses and protection against *Bordetella pertussis* infection after intranasal immunization of mice with filamentous haemagglutinin in solution or incorporated in biodegradable microparticles. Vaccine 13, 455–462.

Chandrasekhar, U., Sinha, S., Bhagat, H.R., Sinha, V.B., Srivastava, B.S., 1994. Comparative efficacy of biodegradable liposomes and microspheres as carriers for delivery of *Vibrio cholerae* antigens in the intestine. Vaccine 12, 1384–1388.

Chang, A.-C., Gupta, R.K., 1996. Stabilization of tetanus toxoid in poly(DL-lactic-co-glycolic acid) microspheres for the controlled release of antigen. J. Pharm. Sci. 85, 129–132.

Cleland, J.L., Powell, M.F., Lim, A., Barron, L., Berman, P.W., Eastman, D.J., Nunberg, J.H., Wrin, T., Vennari, J.C., 1994. Development of a single-shot subunit vaccine for HIV-1. AIDS Res. Human Retroviruses 10, S21–S26.

Eldridge, J.H., Staas, J.K., Meulbroek, J.A., Tice, T.R., Gilley, R.M., 1991. Biodegradable and biocompatible poly(DL-lactide-co-glycolide) microspheres as an adjuvant for Staphylococcal enterotoxin B toxoid which enhanced the level of toxin-neutralizing antibodies. Infect. Immun. 59, 2978–2986.

Esparza, I., Kissel, T., 1992. Parameters affecting the immunogenicity of microencapsulated tetanus toxoid. Vaccine 10, 714–720.

Franz, J., Hampl, J., Stepanek, J., Tesarik, R., 1996. Immunogenicity of infectious bovine rhinotracheitis virus (BHV1) proteins integrated into ISCOMs or liposomes. Vet. Med.-Czech 41, 213–218.

Gregoriadis, G., 1990. Immunological adjuvants: a role for liposomes. Immunol. Today 11, 89–97.

Hampl, J., Dittrich, M., Franz, J., Reschova, S., Stepanek, J., 1996. Adjuvant activity of linear aliphatic polyester and branched aliphatic oligoester microspheres. Int. J. Pharm. 144, 107–114.

Jeffery, H., Davis, S.S., O' Hagan, D.T., 1993. The preparation and characterization of poly(lactide-co-glycolide) microparticles. II. The entrapment of a model protein using a (water-in-oil)-in-water emulsion solvent evaporation technique. Pharm. Res. 10, 362–368.

Laemli, V.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227, 680–685.

Merza, M., Tibor, S., Kucsera, L., Bognar, G., Morein, B., 1991. Iscom of BHV-1 envelope glycoproteins protected calves against both disease and infection. J. Vet. Med. 38, 306–314.

Morein, B., Sundquist, B., Hoglund, S., Dalsgaard, K., Osterhaus, A., 1984. Iscom, a novel structure for antigenic presentation of membrane proteins from eneveloped viruses. Nature 308, 457–460.

Nakaoka, R., Tabata, Y., Ikada, Y., 1995. Antibody production by administration of biodegradable granules incorporating antigen through different injection routes. J. Control. Release 40, 11–21.

O'Hagan, D.T., Rahman, D., Mcgee, J.P., Jeffery, H., Davies, M.C., Williams, P., Davis, S.S., Challacombe, S.J., 1991. Biodegradable microparticles as controlled release antigen delivery systems. Immunology 73, 239–242.

Thomasin, C., Corradin, G., Men, Y., Merkle, H.P., Gander, B., 1996. Tetanus toxoid and synthetic malaria antigen containing poly(lactide)/poly(lactide-co-glycolide) microspheres: importance of polymer degradation and antigen release for immune response. J. Control. Release 41, 131–145.

Towbin, H., Gordon, J., 1984. Immunoblotting and dot immunoblotting current status and outlook.. J. Immunol. Methods 72, 313–340.

Trudel, M., Nadon, F., Seguin, C., Boulay, G., Lussier, G., 1987. Vaccination of rabbits with a bovine herpesvirus type 1 subunit vaccine: adjuvant effect of ISCOMs. Vaccine 5, 239–243.

Van Binnendijk, R.S., Van Baalen, C.A., Poelen, M.C.M., 1992. Measles virus transmembrane fusion protein synthesized de novo or presented in iscom is endogenously processed for HLA class I- and class II-restricted T cell recognition. J. Exp. Med. 176, 119–128.

Van Drunnen Little-van den Hurk, S., Babiuk, L.A., 1986. Synthesis and processing of bovine herpesvirus-1 glycoproteins. J. Virol. 59, 401–410.

Watson, D.L., Lovgren, K., Watson, N.A., Fossum, C., Morein, B., Hoglund, D.S., 1989. The inflammatory response and antigen localization following immunization with influenza virus iscoms. Inflammation 13, 641–649.

.