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Receptor-Linked Antigen Delivery System

Importance of autologous α2-macroglobulin in the development of peptide vaccine

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SUMMARY: We have hijacked a process of the receptor-mediated endocytosis to transport peptide antigens into antigen presenting cells (APCs) for the purpose of increasing the level of antigen presentation (named Receptor-Linked Antigen Delivery System (R-LADS)). By coupling an endogenous plasma proteinase inhibitor alpha2-macroglobulin (α2M) to a synthetic peptide having a partial sequence of HIV-1 envelope protein, α2M was made to carry the peptide into APCs as a part of the normal α2M cycle, which resulted in an increased production of specific antibodies against the peptide (Mitsuda, S., Nakagawa, T., Osada, T., Shimamoto, T., Nakazato, H. and Ikai, A.(1993) Biochem. Biophys. Res. Commun. 194, 1155-1160). We demonstrate here that this procedure becomes a more efficient tool for antibody production when autologous transporter protein was used. By using murine α2M (mα2M) instead of heterologous human α2M (hα2M) when mice were experimental animals, we were able to dramatically enhance the production level of anti-HIV-1 peptide antibodies and shorten the period which is needed for antibody production. We aim to develop effective peptide vaccines by further improving this system. © 1995 Academic Press, Inc.

Recent advances in peptide chemistry made a large-scale production of chemically defined peptides and their use in biochemical application feasible. The potential application of such synthetic molecules as vaccines depends on their ability to successfully mimic naturally occurring epitopes present in the infectious agents and on their capacity to stimulate the appropriate immune response to induce immunity against the natural infection. It has been shown that synthetic peptides, when administered with adjuvants, can elicit the production of antisera capable of neutralizing or binding to many kinds of viruses (1-3). It is known that these synthetic peptide vaccines are chemically better defined and less toxic than ordinary vaccines and would prevent unintentional side-effects caused by contaminants or irrelevant antigens.

Having selected appropriate peptide sequences, we still have a problem of how to elicit a potent immune response in the experimental animal. This has been a major problem in the development of peptide vaccines, particularly in their application to humans, where the selection of adjuvants is limited by safety considerations. Most synthetic peptides have short sequences of 20-40 amino acids, they are most often used in a coupled form with protein carriers such as keyhole

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limpet haemocyanin (1). By facilitating APCs to incorporate more peptides and offer T cell epitopes contained in the carrier protein to APCs, the carrier protein help elicit an immune response. We have directed our attention to the transporting function of carrier protein and developed a Receptor-Linked Antigen Delivery System (R-LADS) to maximize the level of antigen uptake. In this system we used α2M which has a specific receptor on macrophages and dendritic cells as a delivery protein and were able to enhance antigen presentation by the cells and induce high concentrations of antibodies against the delivered antigen (4-7).

α2M is a high molecular weight proteinase inhibitor widely distributed in the extracellular body space of vertebrates as well as that of invertebrates (reviewed in Refs. 8 and 9). Proteinase cleavage of a peptide bond in a defined region of α2M brings about a dramatic change in the structure of the α2M molecule and emergence of four receptor binding sites on the surface of α2M. In addition, treatment of α2M with methylamine is known to result in a nearly identical conformational change to the one brought about by proteinases. The receptor binding-form of α2M is immediately internalized by cells equipped with α2M receptors such as macrophages, fibroblasts or hepatocytes (10, 11). Utilizing this receptor mediated endocytosis, delivery of a variety of molecules has been studied as well as synthetic peptides with defined epitopes (12-14). In our previous work we used hα2M as a carrier of antigenic peptides (7), but we now consider autologous α2M is more desirable as a carrier of antigens in respect of its lower antigenicity and safety. In this report, we show that when murine α2M was used as an autologous carrier protein, the R-LADS procedure induced a higher level of antibodies production and shorten the period needed for the production.

MATERIALS AND METHODS

Animals and reagents 8 week-old female BALB/c mice and BALB/c murine serum were purchased from Nippon Charles River (Kanagawa, Japan). N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) was obtained from Pierce (IL, USA). Methylamine hydrochloride was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Anti-mouse Ig, peroxidase-linked species-specific whole antibody was purchased from Amersham (Tokyo, Japan). ABTS peroxidase substrate was purchased from Kirkegaard & Perry Laboratories (MD, USA). Radioiodination System, [^{125}I]- were purchased from Du Pont-NEN (MA, USA).

Proteins and peptide Hα2M and mα2M were purified from human blood plasma and BALB/c murine serum, respectively, as described previously (15) by using polyethylene glycol 6000 (16), zinc chelate chromatography (Chelating Sepharose Fast Flow, Pharmacia, Uppsala, Sweden) (17), and gel filtration on Sepharose CL-6B (Pharmacia). The peptide named pU (amino acid residues 308-331 of the outer membrane glycoprotein gp120 of HIV-1 (BH-10) (18-20) with an additional cysteine at the C terminus to aid coupling) was synthesized using solid-phase method with ABI 430A Peptide Synthesizer (Applied Biosystems, Tokyo, Japan).

Cross linking of hα2M and mα2M and pU We prepared hα2M-pU and mα2M-pU conjugate freshly prior to every immunization. Hα2M and mα2M were coupled to pU by ligation through disulfide bonds using SPDP. First, introduction of the 2-pyridyl disulfide moiety into hα2M and mα2M was done by gently mixing 4.86 mg of hα2M or 4.6 mg of mα2M in 2 ml of Dulbecco's PBS and 16μl or 14μl of 20 mM DMSO solution of SPDP. After 30 min at room temperature, purification by Sephadex G-25 gel filtration yielded hα2M and mα2M modified with approximately 25 fold molar ratio of 2-pyridyl disulfide linker. Then a sulfhydryl exchange was allowed to occur between the C-terminal cysteine of pU and the 2-pyridyl disulfide group on hα2M and mα2M by mixing their solutions, generating hα2M-pU and mα2M-pU conjugate respectively. After 1 hr at room temperature the degree of coupling was monitored by measuring the formation of pyridine-2-

thione at 343 nm (21), then half volume of 1M methylamine in 50 mM Tris 150 mM NaCl buffer pH 8.5 was added to change the conformation of α2M. After an overnight incubation at 4 °C, α2M with exposed receptor binding sites was purified by Sepharose CL-6B gel filtration chromatography. The conformational change of the protein was checked by PAGE using 4-15 % gradient native gels (PhastSystem, Pharmacia). The protein bands were stained with Coomassie Brilliant Blue.

Immunization The hα2M-pU and mα2M-pU conjugates dissolved in PBS were administered intraperitoneally to each group of 5 mice. The amount of pU in each inoculation was 25 μg/mouse. For a blank test, we administered PBS to another group of 5 mice. Mice were immunized on weeks 0, 3, 6 and 9 and bled on weeks 2, 5, 8 and 11. The titers of antibodies against pU and hα2M were measured by ELISA using anti-mouse Ig, peroxidase-linked species-specific whole antibody and ABTS peroxidase substrate, as described previously (7).

Cellular receptor binding assay Hα2M treated with methylamine was labeled with [^{125}I] using Radioiodination System. Balb/c 3T3 cells were seeded on 24-well culture plates at a density of 70,000 cells per well in minimum essential medium containing 10 % fetal bovine serum and grown for 48 hr until about 95 % confluent. After 20 min at 4 °C, cells were washed twice with 1 ml of 4°C Hanks' BSS buffered with 10 mM HEPES, pH 7.4 (binding buffer). Incubation media were prepared by adding various concentrations of unlabeled competing ligands (hα2M-pU, mα2M-pU) to 0.5 nM ^{125}I-hα2M in binding buffer with 1 % BSA. The final volume added to the cells was 0.5 ml. After 6 hr at 4 °C, the medium was aspirated and the cells were washed once with 1 ml of binding buffer containing 1 % BSA and twice with binding buffer alone. Cells were dissolved in 0.5 ml of 0.1 M NaOH, harvested, and counted for γ-radioactivity. Experiment was performed in duplicate and the results were averaged.

RESULTS AND DISCUSSION

We have used hα2M and mα2M as a transporter of a HIV-1 derived synthetic peptide to APCs. The molar conjugation ratio of pU to α2M varied among preparations and the average was approximately 25. Hα2M and mα2M were exposed to methylamine to generate their receptor binding sites after their coupling with pU. The purity of these conjugates was checked by native PAGE (Fig. 1). We previously showed that treatment with methylamine induced an increase in the relative mobility of hα2M-pU as well as that of native hα2M. No such band shift was observed in the case of mα2M. Before and after treatment with methylamine the hα2M and mα2M which bore pUs had a low relative mobility than native hα2M and mα2M.

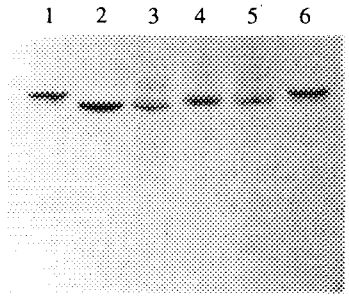

Fig. 1. Native -PAGE analysis of native and modified hα2M and mα2M. Native hα2M (lane 2), native mα2M (lane 3), hα2M-pU (lane 4) and mα2M-pU were charged on 4-5 % gradient native polyacrylamide gel after incubation with methylamine. In contrast, hα2M without methyl amine treatment was charged on lane 1 and 6. After PAGE the gel was stained with Coomassie Brilliant Blue.

Hα2M-pU and mα2M-pU conjugates in PBS and PBS alone were administered to different groups of mice and the titer of antisera against pU was measured by ELISA. We first expected that mα2M could not induce antibodies against pU as well as hα2M, because autologous protein has less T cell epitopes than heterologous one by T cell selection. However, Fig. 2 showed that both carriers induced an immune response against pU. It is important to note that mα2M-pU induced a much higher production level of antibodies against pU than hα2M-pU in mice. It indicated that use of an autologous transport protein is important to get a maximum level of antibody production in R-LADS using α2M. It is likely that other receptor mediated carrier proteins such as C3b (22) and transferrin (23) behave similarly.

Although hα2M-pU and mα2M-pU were effective to produce high concentrations of antibodies against pU in mice, they also induced high concentrations of antibodies against α2M (Fig. 3). Because a small amount of murine immunoglobulin contamination in purified mα2M was detected by anti-mouse Ig antibody linking peroxidase in ELISA, the antisera titer against hα2M was measured. Hα2M induced an almost as same titer of anti-α2M antibodies as mα2M. A small left-ward sift of the graph would result from that the cross reaction of anti-mα2M antibodies against hα2M is weaker compared to reaction of anti-hα2M antibodies against hα2M. Mα2M which did not carry any pU also induced anti-α2M antibody (data not shown). It shows that an autologous carrier protein does not necessarily suppress antibody production against the carrier itself. It was also reported that treatment with both natural and recombinant-derived cytokine may result in anti-cytokine autoantibody formation (24, 25). It is widely accepted that the production of antibodies against autoantigens may result in autoimmune diseases (26). To avoid this problem

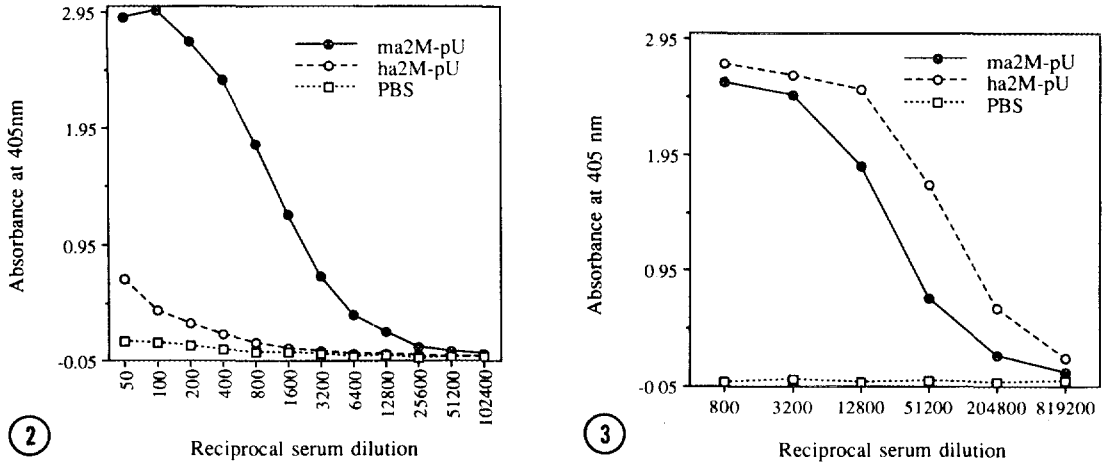

Fig. 2. Titration of anti-pU response elicited in mice immunized with hα2M-pU, mα2M-pU and PBS. The sera tested were obtained on week 8 after immunization on weeks 0, 3 and 6.

Fig. 3. Titration of anti-α2M response elicited in mice immunized with hα2M-pU, mα2M-pU and PBS. The sera tested were obtained on week 8 after immunization on weeks 0, 3 and 6.

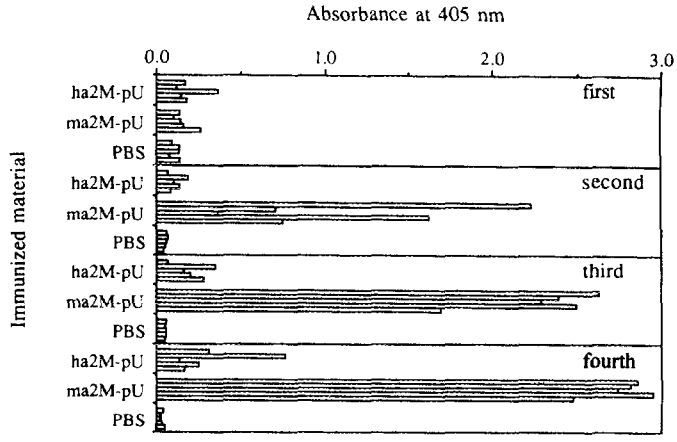

Fig. 4. Titration of the levels of anti-pU antibodies elicited in each mouse administered with hα2M-pU, mα2M-pU and PBS according to the immunization regimen described as follows. The sera were obtained on week 2 (first), 5 (second), 8 (third) and 11 (fourth) after immunization on week 0, 3, 6 and 9 and tested at a 1/200 dilution.

we need a further modification of α2M to block auto epitopes or application of the purified receptor binding site of α2M as a carrier to reduce auto epitopes.

Fig. 4 showed anti-pU antisera titer of individual mice after each immunization. While hα2M-pU did not induce the production of specific antibodies until a third immunization, mα2M-pU induced a high titer of specific antibodies after a second immunization. After a third immunization all mice administered with mα2M-pU induced a high level of specific antibodies. It indicates that an autologous transport protein can not only increase the production of specific antibodies but also reduce the immunization time needed for antibody production.

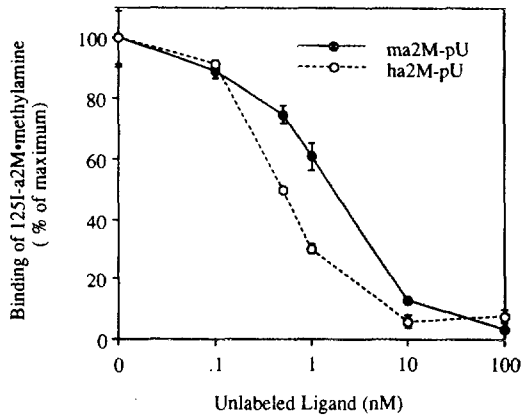

Fig. 5. Binding of hα2M-pU and mα2M-pU to murine α2M receptor on Balb/c 3T3. The ability of hα2M-pU and mα2M-pU to prevent binding of ^{125}I- labeled hα2M treated with methylamine is compared. One hundred percent binding was 837 cpm above a background of 22 cpm in mα2M-pU case.

Since it was possible that the higher level of immune response observed with mα2M-pU was due to higher affinity of the protein to its receptor, we compared the affinity of mα2M-pU and hα2M-pU toward murine receptor as described in the previous section. Fig. 5 showed that competition of ^{125}I-labeled hα2M (206 cpm/ng) with hα2M-pU and mα2M-pU. In the competition both hα2M-pU and mα2M-pU inhibited the binding of ^{125}I-labeled hα2M well. Despite of being an autologous ligand, the affinity of mα2M-pU was somehow lower than that of hα2M-pU. It is in accordance with a previous observation that the clearance of hα2M treated with methylamine from murine circulation was more rapid than that of mα2M with methylamine (27). In the same report it was also noted that 25 % of mα2M was not cleared after a prolonged period. It is possible that being slow to be internalized, mα2M-pU increased its chance to encounter specific B cells against pU. Anyway differential affinities between ligands and receptor did not explain the difference in the level of antibody production.

It should be noted that the fast form of α2M is internalized by an assortment of cells including macrophages, fibroblasts, hepatocytes and neurons (10, 11). The ratio of internalized α2M by each type of the above mentioned cells would depend on the administration sites of the receptor binding form of α2M. Production of antibodies against delivered vaccines, therefore, would vary according to administration sites. In this report we utilized the normal endocytosis cycle of α2M as an artificial antigen delivery pathway, but it is conceivable for α2M to have such a function originally in vivo. Once inflammation occurs in a tissue, macrophages there enhance the α2M receptor expression level (28) and α2M might act as a delivery protein of foreign antigens to the APCs (4). There occurs a high concentration of exogenous as well as endogenous proteinases that can help antigens bind to α2M.

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