CONSTRUCTION OF CHIMERIC IMMUNOGENS: BIOACTIVE FRAGMENT OF HUMAN IL-1 β OR POLYTUFTSIN (PT) CAPABLE OF ELICITING IMMUNE RESPONSES TO HIV PEPTIDES.

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ABSTRACT

In this study, we have examined the effect of linking of bioactive fragment of human IL-1 β (163-171) or polytuftsin (PT, a synthetic polymer of natural immunomodulator "tuftsin") with synthetic peptides of HIV on the induction of immune response to the synthetic peptides. A panel of synthetic peptides representing defined region of gp41, gp120 and gag were used as antigens. Immunomodulators linked peptides (i.e. peptide-IL-1 β or peptide-PT) or peptide dimers were employed for immunization in Balb/c mice. Mice immunized with the peptide-immunomodulator develop effective T-cell proliferation, *in vitro* cytokine release and higher antibody production, but not with peptide dimers. We also found that peptide-immunomodulators induced high level of IgG2a antibody production. Furthermore, there was a positive correlation between the levels of cytokine (IL-2 & IFN- γ) and IgG isotype production. Thus it would appear that incorporation of IL-1 β fragment or PT selectively enhances the Th1 type response to these peptides and may therefore be important for virus neutralization and clearance. However, the effect of IL-1 β fragment was found to be more pronounced than polytuftsin. Such an approach may provide effective vaccination against other infectious diseases.

KEY WORDS: Human interleukin-1 β , polytuftsin, Th-cells, cytokines, chimeric immunogens

Introduction:

Immunization with an antigen containing protective B and T-cell determinants which can elicit potent and broad neutralizing antibodies against HIV proteins, would seem to be required to achieve vaccination against HIV. Increasing evidence suggests that live viral vaccines are unacceptable in the context of HIV disease as the virus can establish the latent infection by various mechanisms. In addition, live or dead virus contains structures that produce deleterious effects, such as enhancing antibodies, which increase the

viral infectivity (1) and suppressing the generation of antigen specific T-cell clones (2). The synthetic peptide vaccine approach for HIV has generated considerable interest for directing protective neutralizing antibodies to the specific epitope of viral or bacterial proteins.

Synthetic peptides have been limited by the shorter half life, less immunogenicity and by the fact that they are MHC-restricted. These limitations of synthetic peptides can potentially be overcome by immunizing peptides conjugated with carrier molecules, incorporating with promiscuous T-cell determinants (3) or in the presence of cytokines (4). Given the limitation of peptide carrier approach, the present study was undertaken to develop an alternative method to induce better immune response against the conserved HIV peptide sequences.

In order to induce optimal immune response to synthetic peptides, bioactive fragment of IL-1 β or polytuftsin (PT) was chosen as an

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immunomodulator, because of its biological properties. IL-1 β fragment lacks the pyrogenic property and mimics the immunostimulatory capacity of the native molecule (5). Secondly, IL-1 β is a first co-stimulatory signal produced during antigen induced T-cell proliferation. On the other hand, polytuftsin has the same biological property as that of monomeric tuftsin with an added advantage of higher half life (6). The most immunogenic HIV proteins like gp41, gp120 and gag have been extensively studied and neutralizing epitopes are well defined. Hence, a panel of highly purified and conserved sequence of synthetic peptides of HIV were used as model peptides in the present study. In HIV infected individuals these monomeric and dimeric peptides detected the anti-HIV antibodies (7).

We report here that when used for immunization HIV peptides linked with immunomodulators (i.e. IL-1 β fragment or PT) modulate the humoral and cellular response and enhance the cytokine production. IgG isotypes were analyzed as they not only influence the biological properties of antibodies but also reflect the development of T-helper subsets involved in regulating the immune response. The immunostimulatory capacity of IL-1 β was always found to be superior as compared to polytuftsin. This approach may prove useful to increase the immune response against poorly immunogenic synthetic peptides.

MATERIALS AND METHODS

Mice: Inbred mice of BALB/C/H-2^d haplotype 4 to 6 weeks old were obtained from the breeding facility of National Institute of Immunology, New Delhi, India. All the experimental groups consisted of 5-6 mice.

Peptide synthesis and purification: Three peptides from conserved regions of gp41, gp120 and gag proteins and bioactive fragment of human IL-1 β (163-171) were synthesized by Merrifield solid phase methods (8). These sequences were selected from HIV-MN strain. The crude peptides were purified by gel filtration followed by high pressure liquid chromatography (HPLC). The purity was found to be more than 90% by HPLC and amino acid analysis.

Bioassay for Interleukin-1 β : The bioactivity of human IL-1 β peptide was assayed by mouse

thymocyte proliferation assay according to the method of Antoni et. al. (5).

Bioassay for Polytuftsin: The phagocytic assay was performed using heat killed *Staphylococcus aureus* with monocytes/macrophages derived from normal individuals to assess the bioactivity of polytuftsin (9).

Construction of Immunogen: HIV peptides were chemically linked to bioactive fragment of human IL-1 β or polytuftsin using double step glutaraldehyde method (10). At the end of the coupling, peptide stoichiometry was found to be 1:1 with peptide IL-1 β and 10:1 with peptide-PT which was determined by amino acid analysis. Peptide dimers were made under identical conditions.

In Vivo immunization: Each group of mice was immunized subcutaneously in the foot pad with 50 μ g of various peptide dimers and peptide conjugates in complete Freund's adjuvant (CFA) (1:1 ratio) in a final volume of 50 μ l. Boosters (25 μ g of antigen in 25 μ l) were given in incomplete Freund's adjuvant (IFA) on day 21 and 28. The animals were bled on day 27 and 37. Sera were separated and stored at -70°C till use. Preimmune sera were collected before immunization.

Measurement of anti-peptide antibodies: Peptide specific antibodies in mice sera were detected by ELISA. Microtiter plate wells (Immulon-II, Dynatech, VA) were coated with 100 μ l of respective peptide antigen (1 μ g/well) dissolved in carbonate buffer (0.05 M, pH 9.6) over night at 4°C. After blocking the nonspecific sites with 2% milk powder, individual mouse serum (peptide-IL-1 β or peptide-PT conjugate immunized mice sera were used at 1:100 dilution and peptide dimer immunized mice sera at 1:25 dilution) was added and incubated at 37°C for 1hr. The antigen-antibody complex was detected using antimouse IgG-HRPO in the presence of H₂O₂ and OPD and the absorbance was read at 490 nm after arresting the reaction. The values are expressed after subtracting the values obtained from the sera.

IgG-Isotype assay: IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) were measured from individual mouse sera using the isotyping kit (Sigma Chemical Co. USA) according to the manufacturer's instructions.

Antigen induced T lymphocyte proliferation: Mice were immunized with 25 μ g peptide dimer alone

or peptide-conjugates (i.e. peptide-IL-1 β or peptide-PT) emulsified with CFA (1:1 ratio, 50 μ l) in the foot pad. On day 8, the animals received a booster with 12.5 μ g of relevant antigens in IFA. On day 12, the mice were sacrificed, spleen was removed and single cell suspension was made in RPMI medium. Cells were then plated (B-cells were removed by panning) at a density of 2×10^5 cells/well, in RPMI medium containing 2 mM L-glutamine, penicillin (50 IU/ml) and streptomycin (50 μ g/ml) supplemented with 5% fetal calf serum (FCS). The cells were stimulated with respective antigens in triplicate wells at three different concentrations (1, 5 and 10 μ g/ml) to give a final volume of 200 μ l. After 72 hr, each well was pulsed with 0.5 μ Ci of 3 H thymidine (specific activity 6.7 Ci/mmol) and the cells were incubated for further 18 hr. Cells were harvested and thymidine incorporation was determined by liquid scintillation counter. The geometric mean counts per minute for each set of triplicate wells were calculated. The counts did not exceed more than 2000 CPM in cells from immunized mice in the absence of antigen (background). The values are expressed after subtracting the values obtained from the background.

Cytokine measurement: 2×10^5 cells/well were plated in 96 well culture plate. Cells were stimulated with 10 μ g/ml of respective chimeric immunogens as well as peptide dimers. Culture supernatants were collected after 48 hr to measure the cytokine levels. IL-2 and IFN- γ concentrations were measured in pooled culture supernatants from the duplicate wells by sandwich ELISA using

commercially available kits (Genzyme) according to the manufacturer's instructions.

Statistical methods: Kruskal wallis, Multiple range tests and Wilcoxon rank sum test (Wilcoxon) signed rank test) were performed to calculate the level of significance.

RESULTS

Bioassays: We assessed the ability of IL-1 β bioactive fragment to induce proliferation of immature mouse thymocytes in the presence of suboptimal concentration of PHA. We observed that IL-1 β bioactive peptide enhanced proliferation in a dose dependent manner only in the presence of suboptimal concentration of PHA (2 μ g/ml). This observation is consistent with others reported in the literature (data not shown). The phagocytic assay was performed using heat killed *S. aureus* with monocytes/macrophages derived from healthy individuals to assess the bioactivity of polytuftsin. We found that PT is biologically as active as that of monomeric tuftsin. Interestingly, PT is biologically more active than monomeric tuftsin in terms of molar concentration (data not shown).

Humoral Response: Chimeric immunogens and peptide dimers were employed for immunization in BALB/c mice and the immunogenicity assessed by measuring total IgG and IgG subclasses. The observation recorded in Figs 1a&b show that immunization with chimeric immunogen (i.e. peptide-IL-1 β or peptide-PT) produced significantly ($P < 0.05$) higher levels of anti peptide antibodies

Fig 1 (a-b) Chimeric immunogen elicits anti-peptide antibodies. Sera were obtained from BALB/c mice immunized either with chimeric immunogen or peptide dimer alone. These animals were boosted on day 21 and 28, and sera were collected on day 27 and 37. Chimeric immunogen immunized mice sera were used at 1:100 dilution and peptide dimer immunized mice sera at 1:25 dilution.

after primary and secondary immunization. The antibody produced immunization was found to be always significantly ($p < 0.05$) higher than that produced following primary immunization. The predominant IgG subclass observed with chimeric immunogen was IgG2a (Figs. 2a & b) irrespective of the peptide used for immunization. On the other hand, animals which received peptide dimer showed low levels of IgG subclasses and were comparable. No statistical difference was observed between primary and secondary immunization. These findings indicate that the IgG2a subclass is predominant when peptides were used for immunization either in the presence of IL-1 β or PT. The interesting observation is that the effect of IL-1 β is more pronounced than PT in terms of IgG2a production.

Cellular response: The chimeric immunogens and peptide dimers were used to induce the T-cell proliferation of spleen cells of BALB/c mice. Lymphocytes were prepared from individual mice immunized with various antigens. The cells were stimulated *in vitro* with respective antigens in triplicate wells at three different concentrations (1 μ g, 5 μ g and 10 μ g/ml). Maximum proliferation was achieved when lymphocytes were stimulated with 10 μ g/ml antigen concentration. Hence, all the data represented here is with 10 μ g/ml antigen concentration.

In one experiment, mice were immunized with peptide-IL-1 β conjugates and spleen cells were stimulated *in vitro* with relevant antigen as well as peptide-PT or peptide dimers. Significantly ($P < 0.05$)

higher proliferative response was observed when cells were stimulated with peptide-IL-1 β conjugates (Figs. 3a-c). Stimulation of cells with peptide-PT conjugates induced moderate level of T-cell proliferation. The low level of thymidine incorporation was observed in cells stimulated with peptide dimers. The response observed with peptide-PT conjugate was found to be significantly ($P < 0.05$) higher than peptide dimer.

In the next group, animals were immunized with peptide-PT conjugates and spleen cells were individually stimulated with either peptide-IL-1 β or peptide-PT or peptide dimer (Figs. 3a-c). Though the mice were immunized with peptide-PT conjugates, *in vitro* stimulation with peptide-IL-1 β conjugate induces either significantly ($P < 0.05$) higher or equal level of T-cell proliferative response as that of cells stimulated with peptide-PT conjugates.

In the last group, animals were immunized with peptide dimers and spleen cells stimulated *in vitro* with relevant antigen as well as chimeric immunogens. The result shows that peptide-IL-1 β stimulated cells induced higher ($P < 0.05$) T-cell proliferation as compared to cells stimulated with other two antigens. Whereas, peptide-PT and peptide dimer stimulated cells induced more or less equal level of T-cell response (Figs. 3a-c). This observation indicates that IL-1 β bioactive fragment is more effective in inducing immune response either immunization or *in vitro* stimulation as compared to PT. However peptide dimer failed to show similar type of response.

Fig. 2 (a-b) IgG subclasses analysis of antigen specific response in serum of mice immunised with chimeric immunogens or peptide dimers. Antigen specific IgG subclasses were examined in these mice using IgG specific ELISA. Chimeric immunogen immunized mice sera were used at 1:100 dilution and peptide dimer immunized mice sera at 1:25 dilution.

Fig 3 (a-c) Induction of antigen specific T-cells by immunization with chimeric immunogen conjugates or peptide dimer alone. Splenic lymphocytes (B cell depleted) were cultured with peptide PT conjugates or peptide alone for 4 days. During the last 18 hr of incubation, 0.5 μ Ci 3 H-thymidine was added. The amounts of 3 H incorporation was determined by scintillation counts. The data shown are 12 days after immunization; results are from five mice per group and are expressed as the mean $\pm$ SEM.

Cytokines: *In vitro* cytokine profile shows that stimulation of spleen cells with chimeric immunogen induced significantly ($P < 0.05$) higher levels of IL-2 and IFN- γ production as compared to peptide dimer stimulated cells. The magnitude of IL-2 and IFN- γ production was significant ($P < 0.05$) with peptide-IL-1 β conjugates as compared with peptide-PT conjugates (Figs. 4 a&b).

Discussion

The objectives of the present study were three fold to: (i) synthesize and characterize HIV peptides, (ii) construct the chimeric immunogens

Fig 4 (a-b) Profile of IFN- γ and IL-2 production by splenic lymphocytes from mice immunized with chimeric immunogen or peptide alone. To examine levels of IFN- γ and IL-2 production, lymphocytes were cultured (2×10^5 cells/well) in the presence of respective antigens (10 μ g/ml concentration which induced optimum T-cell proliferation). Culture supernatants were harvested after 48 hr and then analyzed by the respective cytokine specific ELISA. The data shown are 12 days after immunization.

between HIV peptides and immunomodulators and (iii) evaluate the immunogenicity of chimeric immunogens. The observation of the present study shows that chimeric immunogens are more immunogenic in nature. They can stimulate strong antibody responses following both primary and secondary immunization. The higher antibody response seen with peptide-IL-1 β conjugate could be explained by the direct action on T and B-cells or IL-1 β activated T-cells may be expressing co-stimulatory molecules on the cell surface, which further may activate B-cells to produce antibodies. Peptide-IL-1 β can induce B-cell proliferation and differentiation into antibody producing cells. The present data is in agreement with the findings of Rao and Nayak (11). It also was shown that administration of antigen with IL-1 β can allow genetic non responder to respond (12). The IL-1 molecule and bioactive fragment (163-171) can restore normal immune functions in immunologically depressed and genetically immunodeficient mice (13). The present findings are consistent with the likelihood that immunization with peptide conjugated IL-1 β fragment leads to activation of T-cells which in turn help B-cells to produce antigen specific antibodies.

Trudelle et. al. showed that PT coupled HBV synthetic peptides induced antigen specific primary and secondary immune response (14). Our previous studies suggested that in vivo immunization of malarial peptides coupled with PT induced significantly higher humoral responses (15, 16). The role that PT plays in the antibody production is however, unknown. Recently, it has been shown that polytufts in upregulates the MHC-class-II expression on mice peritoneal macrophages (17) whereas, the present study provides evidence that peptide-PT stimulated spleen cells induce IFN- γ and IL-2 production. Upregulation of MHC-II molecules and induction of cytokine could lead to the antigen specific antibody production in peptide-PT immunized animals.

The functional properties of the IgG subclasses suggest that this immunoglobulin could be important in the defense against viral infection, complement mediated lysis of virus or virus infected cells. Hence, in the present study we determined the profile of different IgG subclasses in response to immunization with chimeric immunogen or peptide dimers. The results of this assay show that chimeric immunogen immunized mice produced IgG2a isotype preferentially, both in primary and

secondary booster immunization as compared to peptide dimers. It has been reported earlier that in the murine system the IgG2a switching is under the control of IFN- γ (18). The present study also shows that immunization of mice with HIV peptides conjugated to immunomodulators induced IFN- γ production *in vitro*, resulting in induced IgG2a production. Observation of the present study implies that immunomodulators play a role on IgG isotype switching mechanism. It is well established that isotype response to particular antigen mainly depends on the dose of antigen, adjuvants, carriers and route of immunization. Commonly used carrier proteins such as BSA, TT, DT and KLH tend to induce predominantly the IgG1 isotype in murine system (19). However, in the present study we found that under identical condition, chimeric immunogen and peptide dimer induce different immunological responses, evidencing that the immunomodulator carrier (IL-1 β or PT) is of critical importance in IgG2a switching. Moreover, IgG1 isotype has been found to possess lower protective capacity than the Ig G2a/2b (20). Takayama et. al. reported that LPS derivatives when used as adjuvant and combination with L141 copolymer increased IgG2a & IgG2b production (21). In another study, *Salmonella flagellen* adjuvant was found to be particularly effective in inducing the production of IgG2a & IgG2b isotypes against the haptens and peptide-conjugates (22, 23). A similar pattern of IgG2a isotype responses was observed, when chimeric immunogens were employed for immunization in mice.

We have also investigated antigen specific T-cell proliferative response to chimeric antigen or peptide dimers. Immunization of mice or stimulation of spleen cells with peptide-IL-1 β conjugate resulted in higher T-cell response. IL-1 β fragment directly acts on T-cells either receptor dependent or independent mechanism. In addition, it also could be due to the effect of IL-1 β through release of IFN- γ by expressing high number of I-A antigens on APC. Polytufts in acts on the macrophage and induces co-stimulatory signal IL-1. Macrophage derived IL-1 acts on the T-cells, resulting in higher T-cell proliferation. It has also been shown that PT enhances I-A antigen expression on peritoneal macrophages (17). These combined effects may lead to the activation of T-cells in a better way. This effect may account for the higher immunogenicity of peptide-PT conjugates as compared to peptide

dimer. These observations are in agreement with our earlier findings, that malarial peptide of circumsporozoite of *Plasmodium vivax* when linked to PT, induced higher T-cell proliferation (15). Whereas, immunization of mice with peptide dimers resulted in low level of T-cell proliferation. It could be due to poor association of peptides with MHC molecules or the activation threshold is not sufficient enough to induce co-stimulatory signal which is essential for T-cell activation, proliferation and cytokine secretion, resulting in low proliferative response. The other possible reason for this is that *in vivo* immunization of peptide dimers alone could result in the induction of anergy on T-cells.

The Th1-Th 2 dichotomy is believed to have a counterpart in humans, and the balance between expressed cytokines is of importance in number of clinical situations. For example, a predominant Th-2 type response can be demonstrated for patients with arthritis, asthma and progression of AIDS. Th-1 type of cytokines are associated with recovery from leishmania and viral infections. In the light of these evidences, we tested the effect of chimeric immunogens on the production of cytokines under *in vitro* condition. Significantly higher levels of IL-2 and IFN- γ production were seen when peptides were conjugated with immunomodulators. This observation indicates that the activation of T-cells with chimeric immunogen leads to the induction of IL-2 and IFN- γ production. In macrophage depleted spleen culture, addition of IL-1 can completely restore the IFN- γ producing cell numbers equal to the numbers of T-cells in the presence of normal APC (24). In addition, incubation of spleen

cells with *S. aureus* and IL-1 can induce synthesis of IFN- γ in a dose dependent manner (24). Polytuftsin exerts its immunological response through macrophages. Macrophage derived IL-1 further acts on the T-cells, resulting in IL-2 and IFN- γ production. In literature, it is clearly demonstrated that PT enhances antigen specific immune response. To our knowledge, this is the first report which demonstrates that polytuftsin increases the production of IL-2 and IFN- γ , resulting in enhanced immune response. Hence, it can be concluded that higher T-cell proliferation could be a result of the immunostimulatory capacity of chimeric immunogen. Peptide dimers lack co-stimulatory properties of chimeric immunogens with the net result that negligible amounts of IL-2 and IFN- γ were induced by these peptide dimers.

In this present study we observed that when peptides were linked with IL-1 β , they always induced significantly higher immune responses as compared to peptides linked with polytuftsin. This effect can be attributed to the difference in half life of IL-1 β (166 min) (25) as compared to tuftsin which has shorter half life (60 min) (6). Secondly, tuftsin is slowly released from polytuftsin over a long period of time; monomeric tuftsin activates T-cells indirectly, whereas IL-1 β fragment directly activate the T-cells for a longer period as compared to tuftsin. The difference in the activity of tuftsin and IL-1 β on account of the difference in their half life period could be at the level of final event of immune response. These findings seem to be highly relevant for developing new peptide immunogens using immunomodulators as carrier.

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