5. RESULTS ....
1. SDS-PAGE analysis of *S. dysenteriae* antigens: The protein profile of the *S. dysenteriae* whole cell sonicate (5 X 10^7 cells) contains a complex mixture of proteins ranging in molecular weights from 10 kDa to 210 kDa (Fig.1 lane a) as seen after separation by 12% SDS-PAGE. The supernatant and pellet of the sonicated *S. dysenteriae* cells are also analysed for the protein profiles after centrifugation at 12000rpm and 4°C for 20 minutes. Their protein profiles show that some protein bands are not there when compared to the whole cell lysate. While a single 116 kDa protein is conspicuous by its absence in the sonicated supernatant (lane b), it is the predominant protein present in the pellet (lane c).

2. Immunogenicity of *S. dysenteriae* antigens: To test the immunogenicity of the various antigens of *S. dysenteriae*, the whole cell lysate is injected into BALB/c mice after emulsifying with CFA or IFA. The reactivity of the antisera thus raised after second immunization and after hyper immunization is checked by ELISA (Fig.2A, 2B) and immunoblot (Fig.2C). The immunoblot shows the reactivity of the antisera to the broad range of *S. dysenteriae* proteins ranging in molecular weights from 17 kDa to 210 kDa. The 116 kDa band which is believed to be associated with cell wall of *S. dysenteriae*, is seen as a distinct and immunodominant antigen in the immunoblot. This protein is selected as the specific antigen against which the immunomodulation is to be monitored for the following studies.

3. Immunomodulation by IL-1β *in-vivo* using *S. dysenteriae* antigens (lysate) in BALB/c mice: The complex antigens (*S. dysenteriae* whole cell lysate) are emulsified in a weak adjuvant IFA either alone or with human recombinant IL-1β. 1000IU of IL-1β per BALB/c mouse is injected based on previous
observations. The reactivity of the antisera generated is analysed by their end point titres to 116 kDa antigen (pellet) by ELISA as shown in fig.3A. The mice immunised with *S. dysenteriae* antigens emulsified in weak adjuvant IFA exhibit a good antibody response after secondary immunization as observed from the end point titres. As expected, the end point titre is much higher when a strong adjuvant CFA is used. But, when 1000IU of human recombinant IL-1β along with *S. dysenteriae* antigens emulsified in IFA is used for immunization, it induces a higher end point titre as compared to the antigens emulsified in IFA alone.

The reactivity of the antisera against the different antigens of *Shigella* antigens is checked by immunoblot. As displayed in the fig.3C, the immunoblot illustrates more number of bands with higher intensity in the case of mice immunized with *S. dysenteriae* antigens along with IL-1β as compared to the antigen alone in IFA. To find out whether the increased titres are isotype specific, isotyping of the sera for all the groups of mice is undertaken by ELISA using isotype specific antibodies namely anti-IgG₁, anti-IgG₂a, anti-IgG₂b and anti-IgG₃. The results, as illustrated in fig.3B, indicate that in all the groups, the antibody response is mostly mediated through IgG₁ and IgG₂a types of antibodies with very low or negligible IgG₂b and IgG₃ levels. As observed, the difference in the end point titres of the mice immunised with antigen in IFA alone and antigen with IL-1β in IFA is in their IgG₁ isotype antibodies mostly. There is no significant difference in end point titres of IgG₁ between the mice immunised with *S. dysenteriae* antigens in CFA and with IL-1β in IFA even though there exists some difference in their end point titres to whole immunoglobulins. Thus, the immunomodulation brought about by IL-1β appears to be IgG₁ specific.

4. Stimulation of Prostaglandin E2 synthesis by IL-1β in HeLa cells *in vitro*: The inflammatory property of IL-1β is assayed in an *in vitro* assay by its ability to
stimulate the release of Prostaglandin E2, a mediator of inflammation, from HeLa cells. The amount of PGE₂ released is quantitated by a sensitive radioimmunoassay. A standard curve is obtained using increasing concentrations of standard PGE₂ and equal amount of its ¹²⁵I labelled tracer. As shown in fig. 4, when is used at a concentration of 25 IU/ml, results in the release of PGE₂ (267.5 pg/ml) which is comparable to that produced by 50 IU/ml (277.5 pg/ml) after deducing the control values. The negligible difference in the amounts of PGE₂ released from HeLa cells by two concentrations of IL-1β, indicates that we have reached the saturation even at the lowest concentration of IL-1β used.

5. Selection of exposed domains of IL-1β: The amino acid sequence of the human recombinant interleukin-1β was analyzed by computational methods for prediction of exposed domains as shown in fig.5A,5B. Using the scales for inverted hydrophobicity and hydrophilicity the regions 47-55, 71-74, 85-96 and 136-143 are found to be hydrophilic and therefore likely to be exposed on the surface of the intact molecule. These regions also correspond to the peak regions when the scales of accessibility and flexibility are considered. Only the regions 47-55, 85-100 and 135-145 corresponded to the peak regions in the secondary structure prediction plot indicating thereby that these three regions are likely to be exposed. Though the antigenicity plot does not show the region 47-55 to be antigenic, because of the strong preference for the β-turn structure and all other parameters in this region it is considered. The amino acid sequences of the predicted exposed domains are as given below.

47-55  VQGEESNDK
85-95  VDPKNYPKKKM
136-143 GTKGGQDI

The three-dimensional structures of the human recombinant IL-1β obtained
from the coordinates of X-ray crystallographic and NMR spectroscopic data are as shown in fig.5C,5D. The tertiary structure shows the molecule to be made up of 12 β-strands connected to each other by either short or long loops (Fig.5E). Using the B-factors values from X-ray crystallographic data, the domains predicted by us to be exposed are found to be on the surface of the molecule. These domains are illustrated in fig.6C, 6D. By measuring the interatomic distances, the domains 47-55 and 88-101 are found to be spatially closer to each other (7°A). The N-terminus is found to be closer to the domain 88-101 (5°A) which in turn is closely spaced to the domain 47-55. But, the N-terminus is relatively farther away to 47-55 (12°A) in comparision to 88-101. When the IL-1β molecule was visualized by different angles, the two domains 47-55 and 85-95 are found to be together as depicted in figs.5F-5J.

6. Peptide characterization: To look at the immunomodulation capacity of different domains of IL-1β, we had synthesized two peptides corresponding to exposed loop regions i.e., loop 4 (47-55) and loop 8 (85-97) of human IL-1β. To test our hypothesis that coupling of different exposed domains of the molecule on the basis of their spatial proximity in the tertiary structure of whole IL-1β, may give us a composite peptide with whole molecule like activity we had synthesized manually the composite peptides consisting of the three domains we predicted to be exposed namely, 47-55-GG-88-101, 47-55-GG-120-151 and 4-16-GG-47-55-GG-88-101. A two glycine spacer between different domains was chosen to maintain their discrete identities.

For determining the minimum domain responsible for immunomodulation around the exposed domain 47-55, we had synthesized peptides corresponding to 41-61, 45-61 and 50-66. The point muteins of the domain 47-55 were also synthesized. These point mutations correspond to Val⁴⁷→Asp, Val⁴⁷→Lys.
Gly⁴⁹→Asp, Glu⁵⁰→Ile and Asp⁵⁴→Ile in different peptides. All the peptides were synthesized as the carboxy terminal acids. The purity of all of the above synthesized peptides was analysed by their ability to bind to hydrophobic matrix in reverse phase column by HPLC and were shown to be > 95% homogeneous as shown in fig.6D. The nature of the peptides corresponding to these homogeneous peaks in RP-HPLC were shown to be the peptides of our interest as revealed both by their amino acid analysis and one dimensional nuclear magnetic resonance spectroscopy. As an example the data is shown only for the peptide 47-55 (Fig. 6D,E). The retention time and the concentration of acetonitrile which caused the elution of each of the mutant peptides in RP-HPLC is shown in the following table.

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>% ACETONITRILE</th>
<th>RETENTION TIME (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native 47-55</td>
<td>9</td>
<td>13.25</td>
</tr>
<tr>
<td>Mut-1 (V47→D47)</td>
<td>6.2</td>
<td>9</td>
</tr>
<tr>
<td>Mut-2 (V47→K47)</td>
<td>3</td>
<td>4.5</td>
</tr>
<tr>
<td>Mut-3 (G49→D49)</td>
<td>10.5</td>
<td>13.9</td>
</tr>
<tr>
<td>Mut-4 (E50→I50)</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>Mut-5 (D54→I54)</td>
<td>15.8</td>
<td>18.45</td>
</tr>
</tbody>
</table>

Stimulation of PGE₂ synthesis by IL-1β and its loop domains in HeLa cells in vitro: Using the same assay, the ability of the IL-1β loop peptides to induce inflammation is assayed. The loop fragment 47-55 of IL-1β as visualized from fig.7 is devoid of any inflammatory activity as it fails to induce production of any
PGE₂ from HeLa cells even at a concentration of 100 μg/ml per 10⁵ cells. In contrast, the loop 7 corresponding to 85-96 results in the release of PGE₂ (40 pg/ml) when used at a concentration of 100 μg/ml containing 10⁵ cells. This is about 1/6th of that produced by the whole IL-1β which is known to be a potent inflammator. But when the two loop regions are synthesized as a composite peptide, with a two glycine spacers, it is shown to be slightly inflammatory as compared to the loop 4 at the same concentration tested. It stimulated the release of 5 pg/ml of PGE₂ which is much lesser than that released by the loop 8 fragment. The other composite peptide containing the C-terminal exposed domain, which is also known to be a receptor binding domain of IL-1β, is also shown to be devoid of any inflammatory activity like the loop 4 peptide. Two peptides are used as irrelevant peptides, one being a random sequence of 9 amino acids long and the other derived from P24 of HIV-1 as irrelevant peptides. While the random sequence peptide fails to elicit any PGE₂, the peptide derived from HIV-1 results in the release of PGE₂ (16 pg/ml).

8. Dose titration of the peptide representing the domain 47-55: A dose titration of loop 4 (47-55) is done to decide the optimal dose of peptide to be injected into mice for immunomodulation along with S. dysenteriae antigens. As illustrated in the fig. 8, there is an increase in the antibody response to the antigen with increase in the amount of peptide injected. A 500 nanomolar concentrations is decided from the above observations as the optimal dose of the peptide as the end point titre increased to about three times (1/7000 to 1/20000). A slight inverse relation is observed between the immunomodulating ability and the amount of salts associated with the peptide (acid and desalted forms) (data not shown).
9. Selection of the minimum domain for immunomodulation: The IL-1β whole molecule and the peptides representing exposed domains 47-55 of human IL-1β are tested for their ability to stimulate antibody response against S. dysenteriae antigens in vivo in BALB/c mice. The whole IL-1β molecule stimulates maximum antibody response followed by significantly lower but almost similar type of immunomodulation response by the peptides 47-55, 41-61, 45-61 and 50-66 as measured by ELISA (Fig.9). Immunoblot analysis gives similar type of results except showing that peptide 41-61 is the most immunomodulatory among the four peptides tested on the basis of number of bands visualized.

10. Immunomodulation by point muteins of 47-55: In vivo immunomodulatory activities of five different point muteins was measured. When Val, the most hydrophobic amino acid present in the loop 4 (47-55) of IL-1β was replaced by a hydrophilic amino acid Asp or Lys, it resulted in a significant increase in the end point titres of total immunoglobulin as compared to the native 47-55 fragment (Fig.10 I,II ). The increase in the total Ig was comparable to that caused by whole IL-1β molecule. The addition of a side chain group at position 49 by replacing Gly—>Asp, resulted in the loss of immunomodulation property of the peptide 47-55 as visualized by the end point titres of total Ig. The replacement of conserved hydrophilic residues Glu⁵⁰ and Asp⁵⁴ to hydrophobic Ile also showed immunomodulation that is comparable to that of the native peptide, indicating the non involvement of these residues in the function of the domain 47-55.

The enhanced immunoglobulin synthesis observed with the mutant-1 (Val⁴⁷—>Asp) and mutant-2 (Val⁴⁷—>Lys) were mostly mediated through
lgG, isotype of antibodies while less significant increase is noticed in the lgG₂a isotype as can be seen from fig 10b. Although mutant-4 (Glu⁵⁰→Ile) and mutant-5 (Asp⁵⁴→Ile) show comparable total Ig levels, at the isotype level only the mutant-5 behaves the same way as the native peptide with increase in IgG₁ and IgG₂a. The mutant-4 resulted in lower levels of both IgG₁ and IgG₂a which are comparable to that obtained by mutant-3. However, in all the peptides the IgG₃ levels were inconsistent. An irrelevant peptide of the same size was also analysed in parallel which showed no stimulation.

11. Stimulation of PGE₂ synthesis by point muteins of 47-55: Like the native 47-55 peptide, the mutant 1 and 2 which showed enhanced immunomodulation caused no stimulation of HeLa cells to secrete PGE₂ in vitro indicating that they are non inflammatory in nature. The same was observed in the case of mutant-5, the mutant-3 which showed in loss of in vivo immunomodulation property of 47-55, and mutant-4 showed slight inflammatory activity as illustrated in fig 11. The levels of PGE₂ detected were negligible compared to that secreted by total IL-1β molecule.

12. Immunomodulation by loop domains of IL-1β : The immunomodulating properties of the domain 47-55 were studied with respect to the receptor binding domains of IL-1β. The individual domain 47-55 showed atleast a two-fold increase both in the amount of total immunoglobulin and the end point titre of total Ig in both strains of mice tested, indicating that, this domain is indeed immunostimulatory across different haplotypes, eventhough significantly lower to that of IL-1β whole molecule response. The enhanced antibody response observed against the complex S. dysenteriae antigens is mostly in terms of an increase in the IgG₁ isotype of antibodies as the difference in the IgG₂a type of antibodies is little (Fig.12A). The incorporation
of IL-1β receptor binding domain 121-150 did not significantly enhance the immunostimulatory capacity of the domain 47-55 as the end point titres were comparable in both the cases. The domain 85-96 shown to be involved in receptor binding also showed immunostimulation to \textit{S. dysenteriae} antigens which is same as that of the domain 47-55, in one strain of mice that is tested (BALB/c) as indicated by the end point titre. But when these two loop domains, one immunostimulatory and the other receptor binding, were synthesized as a contiguous peptide separated by a diglycine spacer and tested for any \textit{in vivo} immunomodulation, it resulted in an enhanced antibody response to \textit{S. dysenteriae} antigens which was again seen in terms of mostly IgG\textsubscript{1}, isotope antibodies. This increase was prominent in BALB/c strain of mice than C57BI/6 (Fig.12B). The presence of the domain 4-16, residues of which were known to be critical for the function of the whole IL-1β, along with the composite peptide containing 47-55 and 88-101, resulted in an enhanced antibody response which is similar to that caused by the two domain alone (pep-2) in the BALB/c strain of mice and a two-fold in C57BI/6 strain of mice. The corresponding irrelevant peptides of the same size were shown to be non immunostimulatory.

13. Immunomodulation by IL-1β and its loop peptides \textit{in vivo} using defined antigens: The immunomodulating properties of the above composite peptides were tested against the less complex antigens like Thyroglobulin and defined antigens like 37 kDa Gelonin in two different strains of mice i.e. BALB/c (Fig.13A, 13C) and C57BI/6 (Fig.13B, 13D). When analysed by SDS-PAGE, Thyroglobulin showed a number of bands while gelonin was homogeneous with molecular weight of 37 kDa. In both the cases the antigen emulsified in CFA induced the maximum antibody response which is higher than that of antibody response to \textit{S. dysenteriae} antigens both quantitatively and in terms of end point titre of total Ig. This was followed by antigen
emulsified in CFA along with whole IL-1β. The peptides caused similar immunomodulation. The domain 47-55 alone could stimulate the enhanced specific response to the corresponding antigen by about two-three fold as quantitated and detected by ELISA. But the composite peptide containing the two domains of IL-1β stimulated a better antibody response than that of the peptide containing the three domains and the peptide 47-55 alone to both the antigens. This is in contrast to that of complex S. dysenteriae antigens. Again the enhanced antibody response observed against these defined antigens was mostly IgG1 antibody mediated. Similar patterns were obtained in the C57BL/6 mice.

The use of other defined antigens like Glucose oxidase, Horse radish peroxidase and a synthetic peptide resulted in immunomodulating patterns as above (data not shown).

Immunomodulation by IL-1β and its peptides in vitro: To see the immunomodulating effect of IL-1β in vitro, thymocyte proliferation is monitored in response to IL-1β at a sub-optimal concentration of ConA. A bell shaped curve is obtained when increasing concentration of the mitogen is used to stimulate immature thymocytes (Fig. 14A). From the above stimulation data a sub-optimal concentration of the mitogen is selected for use in the thymocyte proliferation assay of IL-1β. As shown in fig. 14B there is increase in the proliferation of the immature thymocytes when the thymocytes are cultured with IL-1β alone. But, in the presence of an optimal concentration of mitogen and IL-1β there is a six-fold increase in the proliferation of thymocytes as seen by [3H]-Thymidine incorporation.

Similar results are obtained when antigen (KLH or S. dysenteriae whole cell sonicate) primed T cells are stimulated at sub-optimal concentration of the
same antigen (decided from a dose response curve) (Fig.14E, H) in the presence or absence of IL-1β as measured by [3H]-Thymidine incorporation assay. When the antigen primed T cells are cultured with optimal concentration of the antigen and the suppressive dose of Cyclosporin A (CsA), there is a 17-fold decrease in the proliferation comparable to the control level where cells are not treated indicating a complete immunosuppression by CsA. This suppression is overcome when the above cells are treated with IL-1β as displayed in figs. 14D (ConA), 14G (KLH), 14J (S. dysenteriae).

15. Competition binding of IL-1β and peptides to IL-1 receptors on different cell types: The specific binding of increasing amounts of [125I]-IL-1β added to aliquotes of 5 X 10⁵ DXM treated Raji cells is shown in fig 15A. Scatchard analysis of the binding show that Raji cells have IL-1 receptors whose dissociation constant Kd = 10.5 pM for IL-1β (Fig.15B). The same kind of experiments were then performed on the murine thymoma EL4 cell line and the specific binding obtained with increasing amounts of [125I]-IL-1β are depicted in fig.15A. Scatchard analysis showed that EL4 cells have IL-1 receptors whose Kd = 8.4 pM). The effect of dexamethasone acetate on the number of IL-1 receptors on EL4 was determined to be insignificant by specific binding of increasing amounts of [125I]-IL-1β (data not shown). To characterize further the IL-1 receptors on DXM treated Raji cells, competition experiments were performed. Fig.15D (solid line) demonstrates the displacement of receptor bound [125I]-IL-1β by a wide range of doses of unlabelled IL-1β (1-1000 IU). IL-1β efficiently displaced the receptor bound [125I]-IL-1β. Similar experiments were carried out on normal EL4 cells and the results as shown in fig.15D indicate that the bound [125I]-IL-1β could be replaced by unlabelled IL-1β. As a control, similar experiments were performed using Jurkat and
WEHI274 cells which showed few or no receptors for IL-1β.

The ability of IL-1β peptides spanning single or multiple exposed domains to compete with \[^{125}\text{I}\text{-IL-1}\beta\] to bind to IL-1 receptors was then examined. Fig. 15E shows the competition binding of the only peptide i.e., 47-55-GG-88-101 that showed a little binding to the receptors on EL4 cells but do not bind to receptors on DXM treated Raji cells.

16. Internalization kinetics of IL-1β:

There is a two-fold increase in the ligand-receptor binding when the temperature at which the binding is carried out is increased from 4°C to 37°C (data not shown). Similar experiments when conducted using EL-4 cells yielded a 50% increase with increase in temperature. In both the cell lines the presence of sodium azide during the binding resulted in decreased binding of \[^{125}\text{I}\text{-IL-1}\beta\] to its receptors (data not shown).

The effect of Glycine-HCl buffer, pH 2.8 on the removal of surface bound \[^{125}\text{I}\text{-IL-1}\beta\] from Raji cells is studied. While at both 4°C and 37°C in presence of sodium azide more than 80% of the bound ligand can be removed by acid buffer, in the absence of sodium azide at 37°C only about 65-70% of the bound IL-1β can be removed (data not shown).

The internalisation kinetics of the bound \[^{125}\text{I}\text{-IL-1}\] to IL-1 receptors on El-4 cells was monitored and is depicted in fig. 16B. In case of EL-4 cells there is a steady increase in the binding of \[^{125}\text{I}\text{-IL-1}\beta\] to the receptors reaching an optimum by 3 hours following which there is a decline. During the same period the radioactivity that was resistant to acid stripping increased steadily to 62% followed by a slight decline. The internalised \[^{125}\text{I}\text{-IL-1}\beta\] got associated with the nuclei during the same period from 36% to 70%.
Similar experiments are carried out using Raji cells and the results are as shown in fig 16A. Though a similar increase is seen both in the binding and acid resistant $[^{125}]$-IL-1$\beta$ only a small proportion of it got associated with nuclei (33%). As the Jurkat cells showed few or no IL-1 receptors from the specific binding studies, they are processed similarly as control for the internalisation kinetics of $[^{125}]$-IL-1$\beta$. As shown in fig. 16C, there is little binding of $[^{125}]$-IL-1$\beta$ even after 12 hours incubation at 37°C.

17. Circular dichroism study of 47-55 peptide: The CD spectra of the 47-55 peptide and its point mutants recorded in aqueous medium show that none of the peptides have any secondary structure associated with them (Fig. 17A-F). The spectra when recorded in the presence of a helicogenic solvent Trifluoroethanol show no evidence of formation of any $\alpha$-helical structure in all of the peptides except the Mut-1 peptide (Val$^{47}$-->Asp). Even though the intensity of the negative peak at 220nm is very low, it indicates the presence of helical conformation (Fig. 17B).