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CONFIDENTIAL

In vitro immunostimulant activity of FR-91

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1. Purpose

Evaluation of immunostimulant activity of FR-91

2. Method

1) In vivo T-dependent antibody response (using Plague Forming Cells assay)

- i) Mouse was immunized with 0.025-0.3ml/mouse of FR-91.
- ii) Mouse was immunized with SRBC (Sheep Red Blood Cells) antigen and sacrificed by cervical dislocation and excised the spleen out.
- iii) After 4 days, Ig-conjugated SRBC protein(s) was mixed with lymphocytes, among which are specific antibody producing cells, and incubated in a semisolid supporting medium to allow secreted antibody to bind to the erythrocyte surface.
- iv) Complement with specificity against conjugated antibody to SRBC was added to that mixture subsequently.
- v) Clear zone of lysis(plagues) was measured for assays for B lymphocyte activation.

2) In vitro B cell activation (using Plague Forming Cells assay)

- i) Serial diluted FR-91 samples were added to splenocytes, with making final concentration from 100:1(1%) to 10000:1(0.01%)/well.
- ii) Level of T-dependent antibody producing cells was measured after in vitro treatment with or without lipopolysaccharide using Plague Forming Cells assay.



3) In vitro T cell activation (using Mixed Lymphocyte Reaction)

Lymphocyte from two different inbred strains are cultured together, the cells begin to proliferate in response to the antigenic differences on the allogenic lymphocytes. The intensity of this mixed lymphocyte reaction (MLR) can be quantified by adding tritium-labeled thymidine to the culture medium. As the cells proliferate, the radioactive thymidine is incorporated into the DNA of the daughter cells. The extent of proliferation can be determined by harvesting the cells, lysing them, and measuring the amount of radioactive thymidine incorporated into the DNA, which is directly proportional to the level of cell proliferation.

- i) Lymphocytes from two different inbred strains were mixed in the microwell.
- ii) Serial diluted FR-91 samples (100 : 1 to 10000 : 1) were added to the microwell respectively.
- iii) After culture for 4 days, [³H]thymidine was added to the mixture.
- iv) After culture for 18-24hrs, cells from microwell cultures were harvested on filter-paper strip.
- v) [³H]thymidine incorporated into DNA was counted.



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4) Blastogenesis of T-cells

- i) Serial diluted FR-91 samples were added to splenocytes, with making final concentration from 100:1(1%) to 10000:1(0.01%)/well.
- ii) Respectively named microwell was treated with lipopolysaccharide, pokeweed mitogen, phytohemagglutinin, concanavalin A as standard mitogen, making 1 μ g/well as the final concentration.
- iii) Proliferation level of T & B-cells was measured by [³H]thymidine uptake assay.



3. Result

1) Effect on in vivo T-dependent antibody response (using Plague Forming Cells assay) → Fig.1

FR-91 increased the number of T-dependent antibody producing cells dose dependently in sheep red blood cell immunized mouse. According to this result, FR-91 is effective on in vivo immune system related with many kinds of lymphocyte.

2) In vitro B cell activation → Fig. 2 (using Plague Forming Cells assay)

When splenocytes were treated with or without lipopolysaccharide together with FR-91, FR-91 didn't increase the proliferation of B-cells. There were no effects of FR-91 on the proliferation and activation of B-cell.

3) In vitro T cell activation → Fig. 3 (using Mixed Lymphocyte Reaction)

FR-91 strongly increased the proliferation of T-cell, based on the mixed lymphocyte reaction (MLR).

4) Blastogenesis of T-cells → Fig. 4

- i) FR-91 intensively increased immunity of splenocytes without any mitogen.
- ii) There is no synergism with lipopolysaccharide, which is B-cell mitogen.
- iii) There is some increased synergistic effect with pokeweed mitogen, which is T & B common mitogen, at the high concentration.



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- iv) There is synergistic effect with concanavalin A, which is T-cell mitogen.
- v) There is strong synergism with phytohemagglutinin, which is T-cell mitogen.
- ; In result, FR-91 functions as mitogen by itself and has synergistic effect with T-cell mitogen, with no effect with B-cell mitogen. Therefore FR-91 has stimulating effect on T-cell but not on B-cells.

4. Conclusion

FR-91 has the effects on the proliferation and activation of T-cells, but doesn't have no effect on B-cells.

Fig. 1

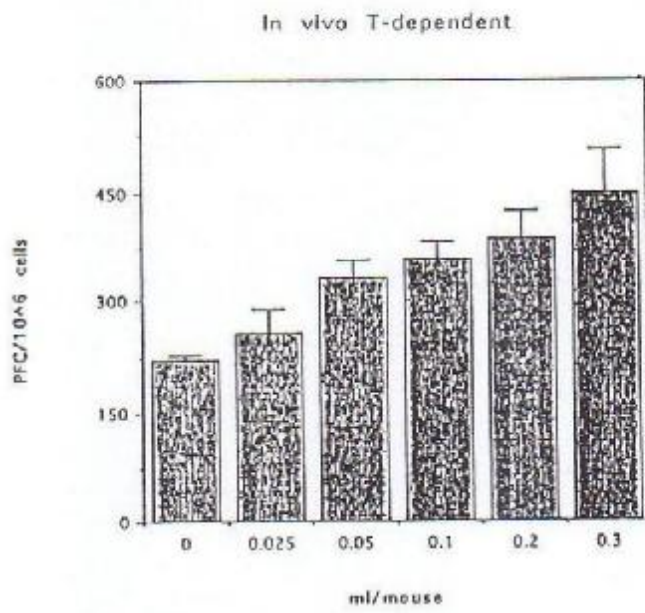


Fig. 2

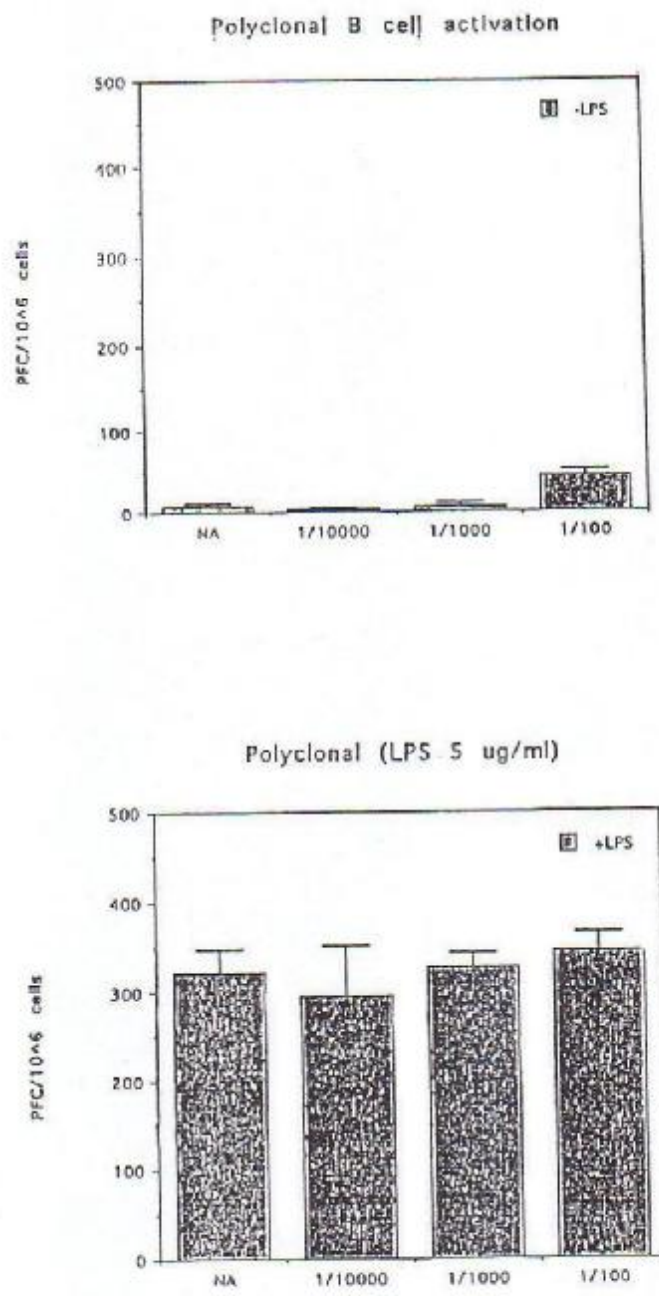


Fig. 3

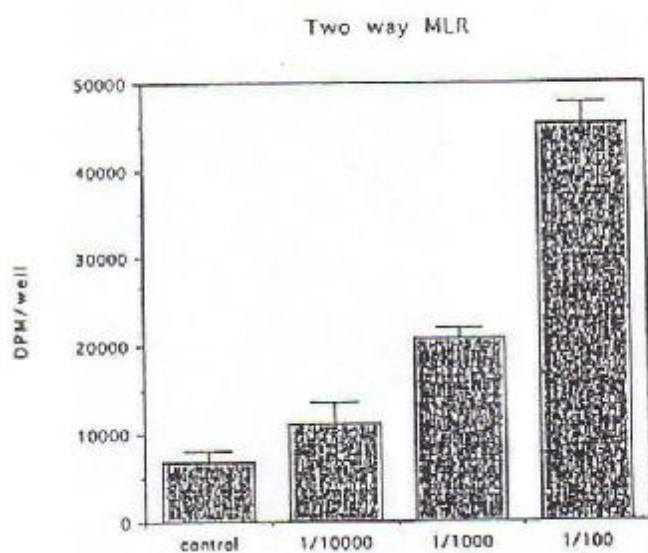
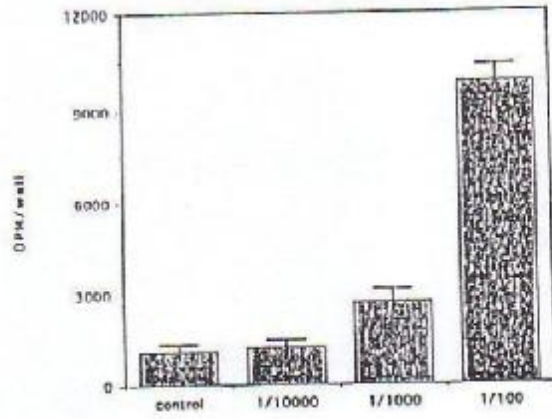
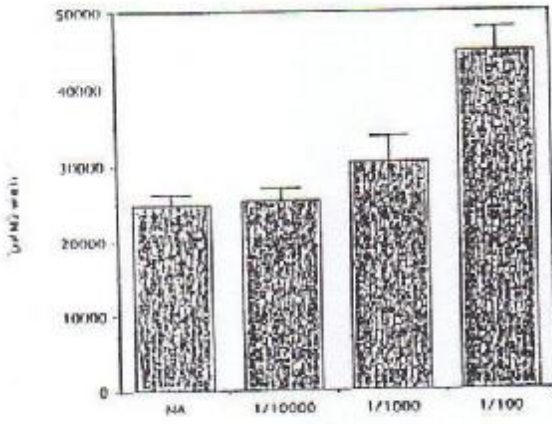


Fig. 4

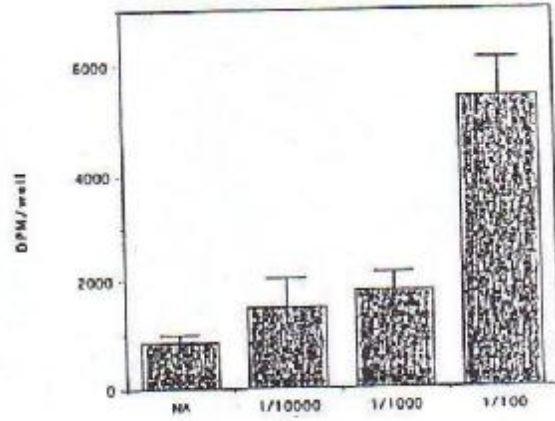
No mitogen



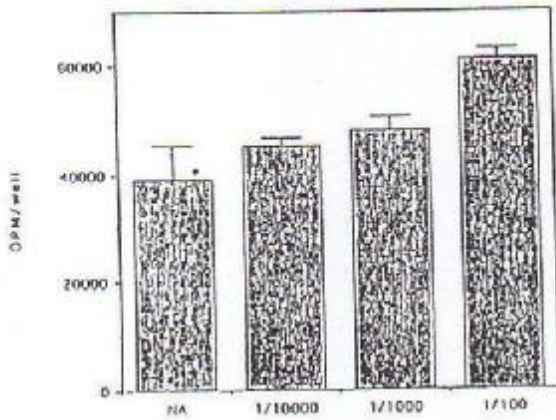
ConA (1ug/ml)



PHA (1ug/ml)



LPS (1ug/ml)



PWM (1ug/ml)

