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SHORT COMMUNICATION

ENHANCEMENT OF ALLO-RESPONSIVENESS OF HUMAN LYMPHOCYTES BY ACEMANNAN (CARRISYN™)

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Abstract — Healing powers have been imputed as being a feature of the gel from the aloe vera plant for centuries. The recent isolation of the active ingredient, acemannan, has made testing of this drug important. Since the drug appears to enhance monocyte function in other experiments, these studies were designed to test the capacity of acemannan to enhance immune response to alloantigen and to test whether the potential enhancement is a monocyte driven phenomenon. Acemannan did not enhance lymphocyte response to syngeneic antigens in the mixed lymphocyte culture (MLC) but importantly increased alloantigenic response in a dose – response fashion (2.6 × 10[illegible] – 2.6 × 10[illegible] M). This effect of acemannan was shown to be a specific response and to concur with concentrations of *in vitro* acemannan achievable *in vivo*. A separate series of mixing experiments demonstrated that acemannan incubation with monocytes permitted monocyte driven signals to enhance T-cell response to lectin. It is concluded that acemannan, the active ingredient of the aloe vera plant, is an important immunoenhancer in that it increases lymphocyte response to alloantigen. It is suggested that the mechanism involves enhancement of monocyte release of IL-1 under the aegis of alloantigen. This mechanism may explain in part the recently observed capacity of acemannan to abrogate viral infections in animal and man.

For centuries magical healing powers have been imputed as a characteristic of the gel from the aloe vera plant. Previously, the capacity of materials from the aloe vera plant to be salutory in clinical practice has been supported most strongly by data relating to the treatment of burns in man and animals. A common feature of the treated lesions is enhanced formation of granulation tissue related to the use of the plant extracts. This observation led to the successful employ of aloe vera extract material in the treatment of wounds (Grindlay & Reynolds, 1986; Cera, Heggers & Robson, 1980; Cera, Heggers & Hagstrom, 1982; Raine, London & Goluch, 1980). The salutory effect of the active moiety of the *Aloe barbadensis* Miller plant, acemannan (Carrisyn™), recently isolated, purified and characterized, in thermal wound healing is felt to be a consequence of activation of mesenchymal cells of the macrophage monocyte lineage which contribute to an enhancement of granulation tissue and to enhanced phagocytic properties of inflammatory cells (McDaniel, Carpenter, McAnalley & St John, 1987).

In further support of the working hypothesis concerning the mechanism by which acemannan may function as an immune activating substance related to the function of cells with phagocytic properties, has been the direct demonstration that acemannan increases the number of spleen cell monocytes in tissue culture and enhances the function of peritoneal macrophages as phagocytic cells against sheep red blood cells (McDaniel *et al.*, 1987). Moreover, compounds similar to acemannan have been postulated to stimulate the release of interferons from lymphocytes, raising the possibility that materials like acemannan may be helpful in treating viral infections. Indeed, the capacity to limit viral infections of acemannan itself has been suggested by recent studies *in vitro* directed against herpes, measles and AIDS virus and *in vivo* against the herpes virus as separate models at high concentration (McDaniel, McAnalley & White, 1987; Carpenter, McAnalley & McDaniel, 1987; McDaniel, Perkins & McAnalley, 1987).

The present study was designed to directly assess the impact of acemannan as an immune enhancer in a model of monocyte – T-lymphocyte cell – cell interaction response to alloantigen presented in the mixed lymphocyte culture. This model tests the

capacity of acemannan to stimulate additional monocyte-macrophage functions in an immunologically relevant model.

EXPERIMENTAL PROCEDURES

Cell preparation

Mononuclear leukocytes were obtained from the peripheral blood of normal, informed and consenting human volunteers under the aegis of a study approved by the Institutional Review Board of the University of Texas Health Science Center at Dallas. Peripheral blood was diluted 1:3 in Hanks' balanced salt solution (HBSS) and layered on top of a Ficoll – Hypaque gradient as previously described by our laboratory (Helderman & Strom, 1977). Cells from subjects known to be disparate for major histocompatability antigens were obtained on each study day to ensure a positive mixed lymphocyte reaction. For specific experiments, more carefully characterized pedigrees of cells which inhabit the mononuclear leukocyte pool were isolated. For the isolation of T-lymphocytes, the standard nylon wool separation technique was used as previously described (Helderman, Ayuso, Rosenstock & Raskin, 1977). The nylon effluent cells contained about 90% pure T-cells. B-lymphocytes and monocyte macrophages preferentially adhere to the column. The adherent population was removed by forcibly pushing media through the column with a plunger. To enrich for monocytes (macrophages), the glass adherence procedure was utilized as described previously (Helderman *et al.*, 1977), giving a population greater than 95% pure.

Acemannan (Carrisyn™)

Acemannan (Carrisyn™), a kind gift from Carrington Laboratories, Irving, TX, was tested in these studies by preparing a 0.5% (*w/v*) solution in RPMI-1640 media, further diluting to the following working concentrations: $2.6 \times 10^{-[illegible]}$ M, $2.6 \times 10^{-[illegible]}$ M and $2.6 \times 10^{-[illegible]}$ M. These working concentrations were used in all of the experiments.

Mixed lymphocyte cultures (MLC)

Unidirectional MLC were set up in flat-bottom, microtiter tissue culture plates (Costar Co., Cambridge, MA). Mononuclear cells isolated by the Ficoll – Hypaque density gradient technique discussed above served as stimulator cells, after exposure to 2000 rad for 30 min in a cesium source (Gammacell, Atomic Energy of Canada, Ontario, Canada). Responder cells, similarly isolated, and stimulators were adjusted to 1.4×10^6 cells/ml. To each well the following was added: 25 μl of acemannan or media (control), 25 μl of RPMI-1640 supplemented with 10% fetal bovine serum and 75 μl of each cell population. Cells were incubated at 37°C in 5% CO_2: 95% air for 6 days. Cultures were pulsed with 25 μl of ^{3}H-thymidine (1 μci/well) for 4 h, after which the cells were harvested and counted. To test the specificity of acemannan on the afferent recognition and response to MHC, additional unidirectional MLC were set up with the agent added just 20 min before the cells were pulsed with ^{3}H-thymidine.

Monocyte – T-cell interaction

Lewis female rat spleens were teased over steel mesh into RPMI-1640 media. Mononuclear leukocytes were collected from the interface of a Ficoll – Hypaque density gradient as previously described above. Monocytes obtained by enrichment on glass petri dishes and adjusted to a final concentration of 10^6/ml, were incubated with varying doses of acemannan or media (control) in a total volume of 2 ml and incubated for 24 h at 37°C. The monocytes were harvested, extensively washed with fresh media and co-cultured with syngeneic T-lymphocytes at a ratio of 10 T-cells: 1 monocyte with the plant lectin phytohemagglutinin (Difco, Detroit, MI) (1:100) for 48 h at 37°C. Cells were harvested over a MASH II (Whittaker, M.A. Bioproducts, Walkersville, MD) placed in fluor, and counted in a scintillation counter (Beckman Laboratories, Chicago, IL). A control experiment was established by incubating T-lymphocytes with acemannan, followed by wash and co-culture with freshly prepared T-lymphocytes, again at 10:1, along with PHA-P.

Specificity

To test the specificity of the chemical agent acemannan, dextran sulfate (mol. wt 60,000), a chemically similar compound was tested. Monocytes, prepared as previously described, were incubated with dextran sulfate (Sigma, St Louis, MO) at final concentrations of $2.6 \times 10^{-[illegible]}$ M, $2.6 \times 10^{-[illegible]}$ M and $2.6 \times 10^{-[illegible]}$ M from a 0.5% (*w/v*) stock in RPMI 1640 or media (control) in a total volume of 2 ml for 24 h at 37°C. The monocytes were harvested, extensively washed with fresh media and co-cultured with syngeneic T-cells at a ratio of 10:1 T-cells:Mφ with PHA (1:100) for 48 h at 37°C. Cells were pulsed with ^{3}H-thymidine, harvested over a MASH II and counted in a scintillation counter.

To test specificity of the responding population used here, monocytes, B-cells and T-cells were isolated from Lewis rat spleens via nylon wool and glass adherence as previously described. T-lymphocytes were incubated with acemannan for 24 h at 37°C. The T-cells were washed and co-cultured at a 10:1 ratio with fresh, syngeneic T-cells with PHA for 48 h. Cells were harvested and counted. B-cells were similarly incubated with acemannan for 24 h and then co-cultured at a 10:1 ratio (*T:B*) with fresh T-cells with PHA for 48 h.

To test the specificity of the immune response measured, monocytes were isolated and incubated with acemannan for 24 h at the three doses. Cells were washed and co-cultured with fresh, syngeneic T-cells (10:1 ratio), T-cells:MΦ with either PHA or lipopolysaccaride (Sigma) for 48 h. Cells were pulsed, harvested and counted in a scintillation counter.

Test of potential intrinsic mitogenicity of acemannan

Human peripheral blood mononuclear cells were isolated over Ficoll – Hypaque. Cells were incubated with acemannan at all three test doses at 37°C, harvested at 24, 48 and 72 h and pulsed for 4 h with tritiated thymidine.

Statistics

All data are described with means ± 1 S.E.M. Statistical analysis was performed by using the Student's *t*-test. Background counts were subtracted out before statistical tests were done.

Purification

The efficiency of nylon wool in separating monocytes from B-lymphocytes and T-lymphocytes has been previously described (Helderman *et al.*, 1987) using cytofluorographic analysis of cell surface analysis. The populations of the T-enriched and monocyte fractions were both greater than 95% pure.

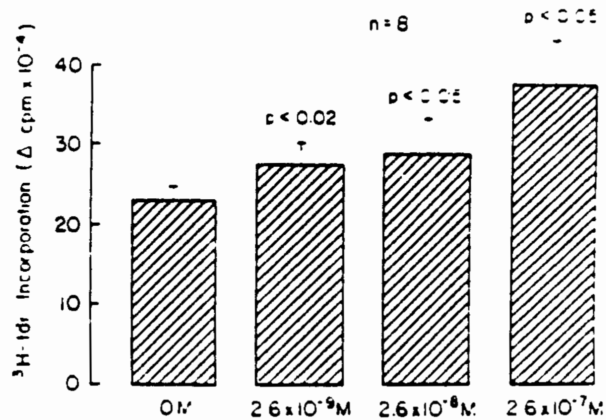

Fig. 1. Effect of acemannan on alloresponse in the mixed lymphocyte response (MLC). Comparison of various doses of acemannan and the amount of DNA synthesis, estimated by incorporation of ^{3}H-Tdr. Drug was added at the beginning of the MLC. The mean ± S.E.M. of eight experiments is shown.

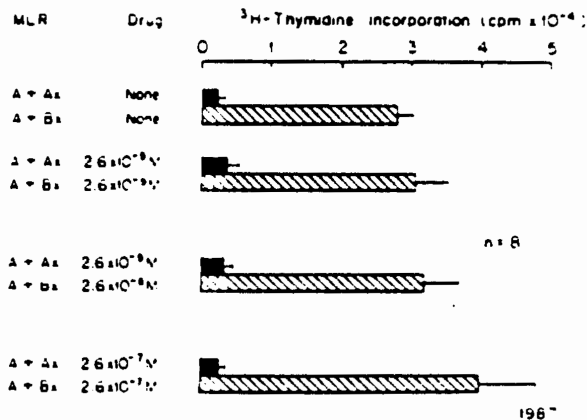

Fig. 2. Effect of acemannan on alloresponse in the MLC. Cultures represented by (A) for syngeneic, (B) for allogeneic, and (X) for irradiated populations. Results are plotted as the counts/min. The mean ± S.E.M. of eight experiments is shown.

RESULTS

Alloantigenic response

Acemannan had no statistically significant effect on syngeneic response of mononuclear cells. When the agent was added at the beginning of mixed-lymphocyte cultures, cells receiving syngeneic stimulation incorporated ^{3}H-thymidine equally in the presence or absence of test reagent at the doses described. In the absence of acemannan these mixed lymphocyte cultures incorporated 2616 ± 1099 counts/min of ^{3}H-thymidine at the end of a 4 h pulse. Although there was a trend upward with respect to the dose of agent added (3281 ± 1355 at 2.6×10^{-9}M, 3742 ± 1670 at 2.6×10^{-8}M and 3828 ± 1978 at 2.6×10^{-7}M), none of these rates of isotopic incorporation into DNA were statistically significantly different.

In contrast to the absence of effect of acemannan on the syngeneic response in the MLC was the effect of the agent on alloresponse in the same immunologic assay. Firstly, acemannan did not

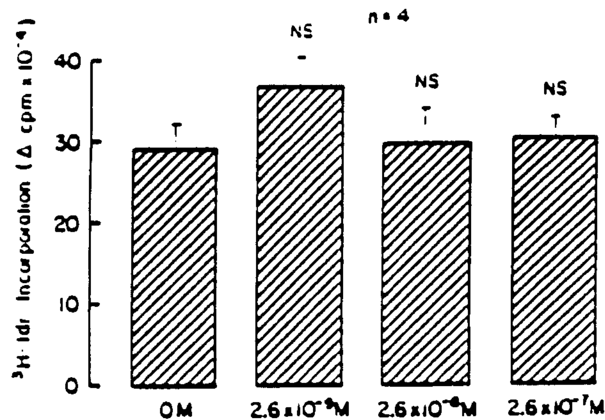

Fig. 3. Effect of acemannan on nonspecific MLC response. Various doses of drug were added to MLC 20 min before addition of ^{3}H-thymidine. The mean ± S.E.M. of four experiments is shown.

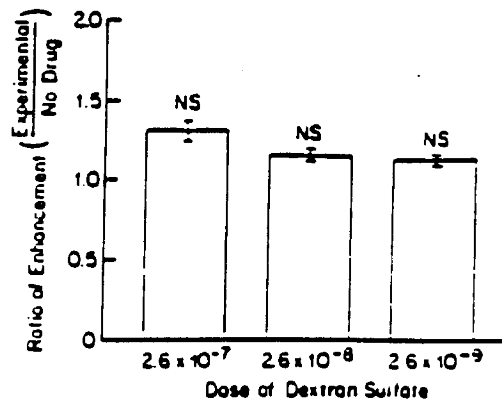

Fig. 5. Effect of dextran sulfate on monocyte – T-cell cooperation. Comparison of various doses of dextran sulfate preincubated with monocytes on T-cell lectin responses after monocyte – T-cell co-culture expressed as the ratio of enhancement (counts/min in experimental well divided by counts/min in control wells).

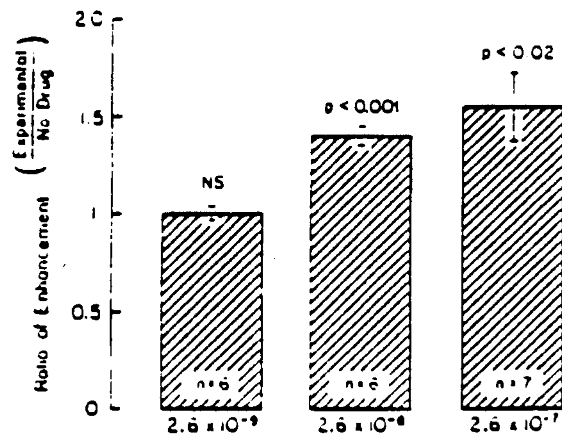

Fig. 4. Effect of acemannan on monocyte – T-cell cooperation. Comparison of various doses of drug preincubated with monocyte on the ratio of enhancement of lectin responses of T-cells after monocyte – T-cell co-culture, expressed as counts/min in experimental wells divided by counts/min in the control wells.

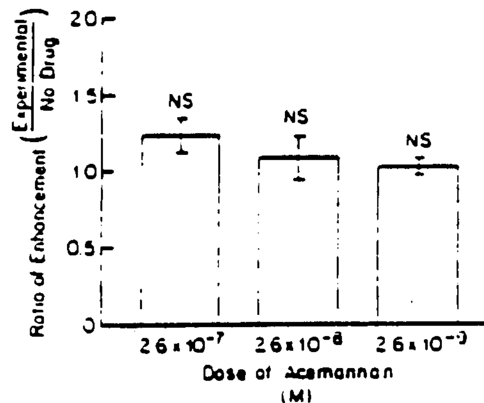

Fig. 6. Effect of acemannan on monocyte – T-cell cooperation in the presence of lipopolysaccaride (LPS). Comparison of various doses of acemannan preincubation of monocytes on T-cell response to LPS expressed as the ratio of on the ratio of enhancement (counts/min in experimental wells divided by counts/min in the control wells).

interfere with the capacity of lymphocytes to recognize and respond to class 2 alloantigenic differences in the mixed lymphocyte culture (Fig. 1); apparent when the syngeneic cultures are compared to the allogeneic response in the presence of the lowest concentration of drug. Secondly, there is a dose-related enhancement of alloresponse by acemannan such that the highest dose tested, 2.6 × 10 M, reflects an almost 60% increase over the culture without an addition of drug. The effect of acemannan on allogeneic stimuli are most clearly seen in Fig. 1, in which the results are plotted as the stimulation index counts/min (the ratio of the alloresponse to the syngeneic response). The dose – response relationship is most convincingly demonstrated, as the enhanced allogeneic response is shown to be significant for each dose of acemannan tested with respect to the no drug condition.

To ascertain whether acemannan exerted a specific effect on lymphocyte alloresponse or a nonspecific effect on ^{3}H-thymidine incorporation, the reagent was added at the conclusion of a 7 day mixed lymphocyte culture, 20 min before the addition of the tracer to the culture. As can be seen in Fig. 3, there was no effect of acemannan when added in this manner as a pulse at the conclusion of the mixed lymphocyte culture. These data support the specificity of the acemannan effect on enhancement

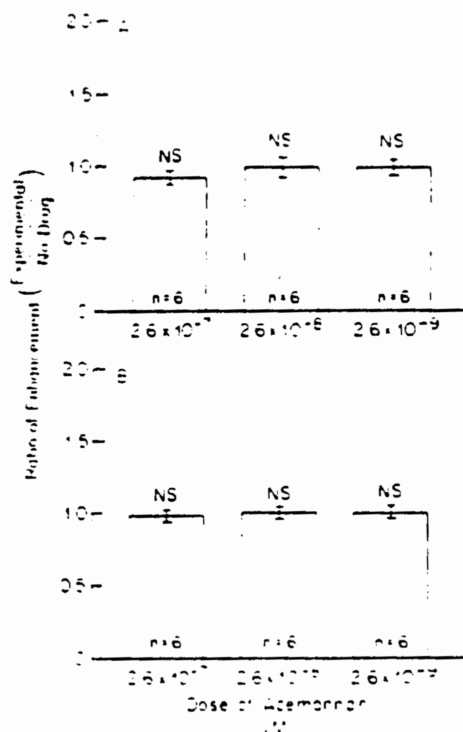

Fig. 7. Panel A: effect of acemannan on T-cell/B-cell cooperation. Comparison of various doses of acemannan preincubation of B-cells on T-cell response to PHA expressed as the ratio of enhancement (counts/min in experimental wells divided by counts/min in the control wells). The mean ± S.E.M. of six experiments is shown. Panel B: effect of acemannan on T-cell/T-cell cooperation. Comparison of various doses of acemannan preincubation of T-cells on T-cell response to PHA expressed as the ratio of enhancement (counts/min in experimental wells divided by counts/min in control wells). Mean ± S.E.M. of six experiments is shown.

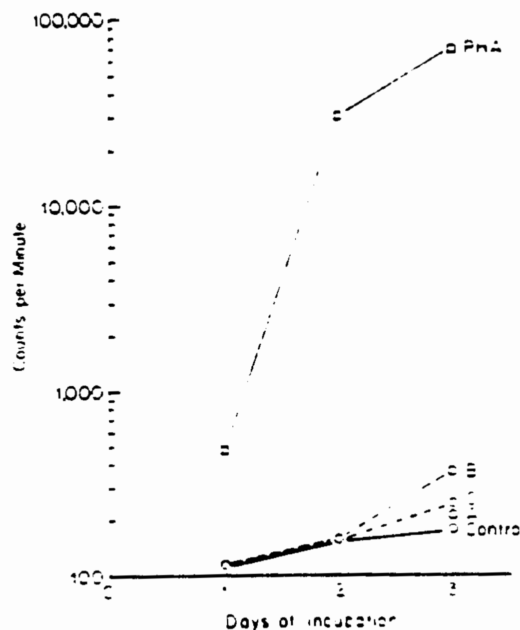

Fig. 8. Acemannan as a potential mitogen. Peripheral blood mononuclear cells were pulsed with ³H-Tdr on days 1, 2 and 3. Results are plotted in counts/min. The doses of acemannan are represented by: □ PHA, positive control; ○ acemannan; A = 2.6 × 10⁻⁷M; B = 2.6 × 10⁻⁸M; C = 2.6 × 10⁻⁹M; control = media. The mean ± S.E.M. of four experiments is shown.

of lymphoid response in the mixed lymphocyte culture.

Acemannan and monocyte – T-cell cooperation

In order to test the hypothesis that acemannan directly stimulates the monocyte responding to alloantigen to provide signal(s) to enhance lymphoid response to antigen and/or mitogen, purified populations of monocytes were incubated with varying doses of drug for 24 h. At the conclusion of the incubation the cells were washed extensively and then co-cultured with T-lymphocytes at a ratio of 10:1, to simulate the natural ratio found in peripheral blood. Co-cultured cells were stimulated with phytohemagglutinin. As can be seen in Fig. 4, the co-cultures with monocytes that have been previously incubated with acemannan had a significantly increased mitogenic response observed in a dose-related fashion.

Specificity

To test specificity of agent, dextran sulfate, a compound chemically similar to acemannan, was examined. In these experiments, monocytes were incubated with dextran, washed and co-cultured with fresh, syngeneic T-cells which had not been exposed to the dextran. T-cell response to the mitogen PHA-P was unchanged by the dextran sulfate, thus the dextran sulfate did not have a similar enhancing effect on monocyte – T-cell lectin response as did the acemannan (Fig. 5).

To test specificity of acemannan to be a nonspecific reagent with respect to the T-cell stimuli examined, monocytes were incubated with acemannan followed by co culture with T-cells and stimulated with the B-cell mitogen, LPS. As seen in Fig. 6, there was minimal T-cell response to LPS basally but no enhancement of this response by acemannan.

To test specificity of the T-cell – monocyte interaction for the acemannan effect, deletion and enrichment experiments were performed in which acemannan was incubated first with T- or B-lymphocytes and co-cultured with freshly isolated, syngeneic T-cells (10:1 ratio) plus PHA. Preincubation with T- or B-cells did not effect a significant increase in T-cell lectin response (Fig. 7).

One can conclude that the acemannan effect is specific for the monocyte and T-cell interaction. Only when monocytes were incubated with syngeneic T-cells was there a dose-related mitogenic response.

Mitogenicity of carrisyn

Human peripheral blood mononuclear cells were incubated with acemannan or PHA (positive control). The cells were pulsed and harvested at 24, 48 and 72 h. At none of the doses tested was a significant mitogenic response observed. The PHA cultures showed a characteristic, mitogenic T-cell response at 72 h (Fig. 8).

DISCUSSION

These studies have explored the capacity of acetylated polymannan, which has recently been isolated and purified from the aloe vera plant (*Aloe barbadensis* Miller), to function as an immunostimulating drug with important clinical consequence. The possible anti-infectious and healing roles of a product(s) of the aloe vera plant has been known since ancient times. More recently, an attempt to formally approach the manner in which aloe vera may be a useful agent in human disease has been undertaken, especially in the face of the recent isolation of the active ingredient.

There is now mounting evidence that acemannan may be an agent capable of delimiting infections to DNA and retro-viruses that cause significant disease in animals and in man. For example, in the animal model, acemannan has reduced feline viral rhinotracheitis (Carpenter *et al.*, 1987). There is additional evidence that acemannan *in vitro* and *in vivo* may be effective against herpes simplex 2 virus, the measles virus and perhaps the HIV (McDaniel *et al.*, 1987). The immunologic mechanism by which the drug may function is as yet unknown. There is some indication that the mechanism may involve enhancement of the monocyte both as a phagocytic cell and as a cell that contributes to afferent recognition of antigen. Studies have shown direct enhancement of phagocytic properties of the monocyte on the one hand and an increase in the absolute numbers of that important cell on the other (McDaniel *et al.*, 1987). There is mounting evidence to support the concept that acemannan enhances the elaboration of the signal substance interleukin-1 by the activated monocyte (McAnalley, unpublished observation).

The studies described in this manuscript were directed specifically at exploring the mechanism by which acemannan may be an immunoenhancing reagent. The mixed lymphocyte culture is an *in vitro* model of the manner in which immunocompetent cells participate in response to antigens of the variety that is necessary for recognition and response to viruses. In this reaction there are important monocyte–T-lymphocyte interactions in order to generate a response to alloantigen. It was this model that was chosen to test the capacity of the drug to function as an immunoactivator.

Our studies clearly demonstrate that acemannan is an important enhancer of the alloantigenic response in the mixed lymphocyte culture in a dose–response fashion, with enhancement at the highest dose tested here about 60% above basal. This represents not only a statistically significant but also a biologically relevant increase in response to alloantigen and may serve as one means by which the drug can aid the response of the organism to viral assault. This effect of acemannan was shown to be specific for the allogeneic stimulus provided the drug did not enhance either basal response to self (syngeneic mixed lymphocyte cultures) or non-specific incorporation of a tracer DNA precursor, ^{3}H-thymidine, when drug was added at the conclusion of the mixed lymphocyte culture.

In a second series of experiments we tested the hypothesis that monocyte–T-lymphocyte interactions may be at least in part responsible for the heightened alloresponse in the mixed lymphocyte culture. In this series of experiments the acemannan was incubated alone with monocytes, after which the treated, extensively washed monocytes were intermixed with freshly prepared, syngeneic T-lymphocytes which had not seen and would not see the drug. In these experiments we demonstrated enhancement of T-lymphocyte responses to the polyclonal mitogen phytohemagglutinin, a second T-cell immune response, at a magnitude equal to the response that we had seen previously in the mixed lymphocyte culture, approximately 55% above baseline with a dose–response relationship.

Curiously, the lowest dose that was tested in our studies which was effective in the mixed lymphocyte culture had no effect in the monocyte experiment. We have no direct explanation for the difference. It is not surprising that the threshold dose may be different for the two models tested, polyclonal response to mitogen and alloantigenic response in the mixed lymphocyte culture. One can also observe that the monocyte experiment is a more stringent test of the effect of acemannan since it presents a treated cell type, the monocyte, to T-cells which then see immune stimulus in the absence of drug. While

alloantigenic response may be due solely or in great measure to acemannan-enhanced monocyte activity the less polyclonal mitogen-enhanced response may be a consequence of an assay of immune stimulations each with a different threshold response to acemannan. To sustain this interpretation of the observed data will require further study.

An array of controls were performed to test the specificity of the acemannan effect on immunoenhancement. Firstly, we examined the specificity of the specific polymer used, acemannan, by choosing to study a chemically similar molecule, dextran sulfate, which has begun to get some press as a potential immunoenhancing agent against AIDS. Utilizing the same protocol with respect to monocyte – T-cell interaction for T-cell lectin response, we could not demonstrate an immunoenhancing effect of dextran sulfate. This allowed us to conclude, with respect to at least one chemically similar molecule, that the acemannan effect was specific for that molecule. Secondly, we tested the specificity of the acemannan effect on at least one non-T-cell response to determine whether the effect was specific for the monocyte – T-cell interaction or totally non-specific. For these experiments we utilized the B-cell lectin lipopolysaccaride. We could not demonstrate that preincubation of monocytes with acemannan led to an enhanced response of T-cells to LPS, allowing us to conclude that the acemannan immunoenhancing effect was specific for T-cell responses. Indeed, we studied two distinct T-cell responses in this work, alloresponse in the mixed lymphocyte culture and lectin response to phytohemagluttinin, both of which were enhanced in a dose – response fashion by the test reagent. Lastly, we tested the specificity of the monocyte – T-cell interaction itself by incubating first B-cells or T-cells with the acemannan, followed by T-cell co-culture. These experiments reveal that only when acemannan was incubated with monocytes and then co-cultured with syngeneic T-cells and pulsed with the lectin phytohemagluttinin did we observe an enhanced lectin response. One can conclude from these experiments that the acemannan effect is specific for monocyte – T-cell interaction and specific for T-cell responses themselves. Lastly, it is possible that acemannan functions merely as a nonspecific T-cell mitogen to explain the enhancement of alloresponse in the mixed lymphocyte culture and to explain in part the monocyte – T-cell interaction experiments. We tested directly the potential mitogenicity of acemannan and found it lacking in this regard. One can conclude once again that the T-cell response is specific for acemannan.

An important question that must be raised is whether the dose of acemannan used in these experiments is clinically relevant. Indeed, the dose range selected was chosen precisely to bracket that concentration of acemannan that could be expected to be achieved in plasma if the drug distributes in extracellular water and is absorbed at the rate of a third of the orally administered dose, figures that were based on previous pharmacologic studies in dogs. The actual concentrations achievable in man also have been shown to be in this range further supporting the potential relevance for clinical practice of these studies.

Additionally, we performed an experiment to determine whether acemannan is itself a mitogen. As illustrated from the time course and the figures, acemannan was not found to be a mitogen, especially when compared to PHA.

In summary, acemannan, the active substance derived from the aloe vera plant which from time immemorial has been felt to be clinically beneficial, has been shown to be an immuno-enhancing agent *in vitro* with respect to allogeneic responsiveness in the mixed lymphocyte culture, perhaps by virtue of its enhancement of monocyte function in its capacity to compete with the T-lymphocyte for antigenic recognition. This drug holds important promise as a clinically useful anti-viral agent.

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