INDUCTION AND REGULATION OF HUMAN PERIPHERAL BLOOD TH1-TH2 DERIVED CYTOKINES BY I_E WATER PREPARATIONS AND SYNERGY WITH MITOGENS

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Recent studies by Lo *et al* [1,2] have reported on the physical properties of water with I_E structures. These structures exhibit a variety of distinct physical and chemical properties as compared to ordinary control water preparations.

We have initiated studies to investigate the effect of I_E water in a biological system in order to establish a physiological role for I_E water.

We investigated the induction and regulation of synthesis of several cytokines, mediators of both the antibody and cell-mediated immune responses to infection and cancer, by human peripheral blood-derived leukocytes and purified subsets. Cytokine secretion was determined by a sensitive and specific Elisa and transcriptional regulation of mRNA by RT-PCR.

We demonstrate with one I_E water preparation provided by ATG that it possesses potent immunomodulatory activities in the absence and presence of suboptimal concentrations of T and B cell mitogens.

The effects were specific for I_E water as no effect was seen with control water preparations from ATG or laboratory-derived water. The following findings were reproducibly obtained with I_E water preparations:

(1) There was significant stimulation of several cytokines by human peripheral blood, namely tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), IL-b, IL-12, and interferon-γ (IFN- γ). The amount secreted by I_E water was very significant, higher or similar to optimal concentrations of mitogen-induced stimulation of cytokines. The secretion of cytokines was also observed with purified subpopulations of lymphocytes and monocytes.

(2) The induction of cytokine secretion by I_E water was a function of both the final concentration of I_E water and the time of incubation. There was a dose-dependent titration of cytokine secretion with different dilutions of I_E Further, we observed a very rapid kinetics of induction of cytokines by I_E water as early as 2 h following stimulation.

(3) In the presence of suboptimal concentrations of the B cell mitogen (LPS) and the T cell mitogen (PHA), we obtained a significant synergistic activity with I_E water but not with control water preparations.

(4) The levels of cytokine induction differed from one individual to another, but the pattern remained the same for all individuals.

(5) In several instances, we observed a specific pattern of cytokine induction by TH1 and TH2 subsets. These two subsets regulate the outcome of several human diseases.

We were concerned about the possibility that I_E water was contaminated with bacterial products, like the lipopolysaccharide (LPS), which is mitogenic and stimulates high levels of cytokines by peripheral blood. We examined the effect of a specific inhibitor of LPS, polymixin B, in the cytokine response by human peripheral blood to both I_E water and LPS. The findings demonstrate that whereas LPS-induced cytokines secretion is completely inhibited by polymixin B, the cytokine response by I_E water was not affected at all by polymixin B. These findings rule out LPS as a potential contaminant in the I_E water preparation.

Altogether, these findings demonstrate that I_E water preparations exert potent and selective immunomodulatory activities on human peripheral blood. Further, I_E water potentiates and synergizes with antigenic stimulation and thus may have an important role both as an adjuvant and/or as a regulator of specific immune responses.

Since different water preparations can be prepared with different I_E crystals, we hypothesize that each might have a distinct and selective pattern of cytokine induction and immunostimulatory activities. The potential benefit of I_E water preparations in human diseases need to be explored.

1 Introduction

Infections (viruses, bacteria, fungi, protozoa and helminths) cause damage to tissues. Body surfaces are protected by skin epithelia and epithelia that line the gastrointestinal, respiratory, and genitourinary tract. Infections take place when pathogens colonize or crossover the epithelial barriers. When pathogens crossover epithelial barriers and begin to replicate in the tissues of the host, the host's defense mechanisms are required to eliminate the pathogens.

The first phase of host defense is innate immunity. Different infectious agents cause markedly different diseases, reflecting the diverse processes by which they damage the tissues. Many extracellular pathogens cause disease by releasing specific toxic products or toxins. Intracellular infectious agents frequently cause disease by damaging the cells that host them. The pathology caused by a particular infection depends on the site in which it grows.

Host immunity can be divided into innate immunity and adaptive immunity. Innate immunity: exposure to infection is measured by host responses that are present at all times. These include epithelial barriers to infection, phagocytosis by monocytes and macrophages, secretion of mediators, and natural killer cells that kill infected cells and cancerous cells. Innate immunity is characterized by lack of specificity for a particular pathogen and lack of memory (responses do not increase with repeated exposure to the same pathogen). Adaptive immunity: it is characterized by the response of specific lymphocytes and the development of immunological memory. Adaptive immune responses are generated by clonal selection of lymphocytes. These antigen-specific lymphocytes, namely B and T cells, result in the production of antibody response and cell-mediated immunity, respectively.

While antibodies are useful in the blood and accessible spaces and can neutralize extracellular pathogens, bacterial pathogens and parasites and virus that replicate inside the cells cannot be detected by antibodies. These can be destroyed by T cell-mediated immune responses.

T cells have been subdivided into two types: cytotoxic ($CD8^+T$) and helper ($CD4^+T$). The T helper is subdivided into TH1 and $TH2^3$. TH 1 cells secrete soluble mediators or cytokines that regulate the antibody response by B lymphocytes. TH2 cells secrete cytokines that regulated delayed-type hypersensitivity and inflammatory responses. The cytotoxic T cells kill infected cells and cancerous cells.

The cytokines activated by pathogens are responsible for directing the immune response towards a preferential TH2-dependent B cell antibody response or a preferential TH1-dependent cell mediated response, or both. Different pathogens are eliminated

preferentially by either antibodies or by cell-mediated responses. Thus, the final outcome of the immune response and their effect in the elimination of infections.
The objective of this study was to initially examine whether IE water cluster preparations, used alone or in combination with antigens, have any effect on the regulation of cytokine synthesis and production by human peripheral blood leukocytes and to determine whether there exists a selective triggering of TH1 vs. TH2 type of cytokines.

2. Materials and Methods

2.1 Monocyte Isolation

This was done as described[4]. Briefly, monocytes were isolated from EDTA-anti-coagulated human peripheral blood using a modification of the Fogelman method[5]. Briefly, white blood cells, WBC, were separated by mixing 10 parts of blood, 1 part of 6% Dextran 500 (Accurate chemical) and leaving the tube standing at room temperature for 40 min. The WBC, after a serial hypertonic treatment with gradually increased osmolality, were laid on monocyte isolation buffer (Nycoprep 1.068) and centrifuged at 600 g for 15 mm. The monocytes (PBM) at and below the interface were harvested, washed once with PBS, and suspended in RPMI with 10% autologous serum for further use. The preparation of peripheral blood lymphocytes (PBL) was done according to Jewett et al. [4].

2.2 Whole Blood Cytokine Induction

Blood drawn from normal donors was sampled into sodium heparin-containing sterile blood collecting lubec (Vamtamer, Becton Dickenson & Co.)[6]. For cytokine induction, 0.3 ml of blood was added to 1.2 ml of RPMI-1640 containing 50% of either control water or I_E water, with or without other indicated treatments. For LPS treatment, the samples were incubated for 18 h, and for PHA stimulation, they were incubated for 48 h, except as otherwise indicated. At the end of the culture, the samples were gently mixed by swirling the test tube, and cell free supernatants were harvested after centrifugation.

For cytokine induction from PBM, PBL and monocytes, 100 µl of cells (10^7 cells/ml) were added to each sample (900 µl of PPMI-1640 containing 50% of control or I_E water) and incubated for 18 h for monocytes, 48 h for PBL, and for 18 h or 48 h for whole blood depending on the stimulation.

2.3 Quantitation of Cytokines

This was done according to our published procedures.

3. Results

We have investigated the effect of I_E water preparations, alone or in combination with mitogens, on the induction of cytokines by human peripheral blood-derived leukocytes and subsets. The cytokines were assayed by sensitive and specific quantitative Elisa methods as described4.

3.1 Effect of I_E Water on Human Peripheral Blood Leukocytes

3.1.1 Spontaneous Induction

We examined the effect of I_E water preparations on the spontaneous induction of several cytokines by human peripheral blood. The final concentration of I_E water was 50% as it was mixed with 2x medium. Table 1 shows a representative experiment whereby TNF-α, IL-6 and IL-12 were measured. Clearly, two different batches of I_E water (I_E #1 and #2) stimulated significant levels of all three cytokines. Control water from the laboratory or from ATG resulted in very low levels of TNF-α (<45 fold) and no IL-6 or IL-12. These findings demonstrate that the culture of peripheral blood in I_E water-containing medium stimulates the secretion of significant levels of several cytokines.

Table 1: IE water-induced cytokine production by whole blood.

Water Preparation	Cytokine (pg/ml)		
	TNF-α	IL-6	IL-12
Laboratory	260	0	8
ATG Control	260	0	9
I_E #1	12190	3906	1041
I_E #2	10735	4797	1190

The laboratory medium (RPMI 1640) was pretreated at 2x concentration. It was used to dilute (1:1) with various water preparations. Whole peripheral blood was diluted (1:5) in the medium and incubated for 18 h at 37°, 5% CO_2 incubator. Thereafter, the cell free supernatants were removed and stored for analysis for cytokines. The results presented are mean of duplicate tests of 3 different experiments.

3.1.2 Titration of Potency of I_E Water

We then examined if the spontaneous induction of cytokines by I_E water is dependent on the final concentration of I_E water clusters present in the culture. Different dilutions of I_E water were made and tested for their induction of cytokines. Table 2 shows a representative experiment for TNF-α and IL-12. Clearly, the secretion of both of these cytokines is a function of the final I_E water concentration. Significant cytokine induction was observed after 10 fold dilutions.

3.1.3 Effect of I_E Water on Mitogen-Induced Cytokine Production

Several mitogens, such as lipopolysaccharide (LPS) and phytohemagglutinin (PHA), stimulate the proliferation and secretion of cytokines by peripheral blood leukocytes. We examined the effect of I_E water on both LPS and PHA-mediated cytokine secretion. Table 3 represents data obtained from LPS-mediated stimulation of peripheral blood. LPS is a potent mitogen for monocytes.

Table 2: Effect of titration of IE water on cytokine production by human whole blood.

Type of cytokine	Cytokine (pg/ml)			
	1:2	1:6	1:18	Non I_E Control
TNF-α	14176	4649	1593	430
IL-12	2405	3405	529	39

The laboratory medium (2x) was diluted with water preparations (1:1) to give a final concentration of 50%. This was diluted three fold and the experiment was Performed as in the legend of Table 1.

*Table 3: Effects of IE water on **LPS-induced** cytokine production by whole blood.*

Water Preparation	Cytokine (pg/ml)		
	TNF-α	IL-6	IL-12
Laboratory	8065	4014	1008
ATG Control	7211	4153	907
I_E #1	13222	4331	1040
I_E #2	12933	4902	1239

The peripheral blood was prepared as in the legend of table 1 except that LPS (10 ng ml) was used for mitogenic stimulation.

Table 4. Effect of I_E Water on PHA-induced cytokine production by whole blood.

Water Preparation	Cytokine (pg/ml)			
	TNF-α	IL-6	IL-12	INF-γ
Laboratory	131	19	40	703
ATG Control	117	19	41	703
I_E #1	13197	2414	1228	1043
I_E #2	10204	2557	1206	2230

The peripheral blood was prepared as in the legend of Table 1 except that the cultures were stimulated with PHA (5 µg/ml) and incubated for 48 h before harvesting.

Clearly, while control water shows significant production of TNF-α, IL-6 and IL-12 by LPS, the combination of LPS and IE water resulted in significant potentiation of the secretion.

Table 4 shows representative results of cytokine secretion by PHA stimulated human peripheral blood. PHA is a potent mitogen for T cells. The data in Table 4 show that PHA does not stimulate, as expected, significant TNF-α, IL-6, and IL-16 (monocytes derived) but stimulate T cell-derived IFN-γ. The combination of I_E and PHA potentiate the secretion of IFN-γ.

These findings demonstrate that combination of I_E water with mitogens potentiate the secretion of selective cytokines by peripheral blood.

3.1.4 Synergy

We investigated whether I_E water can potentiate the cytokine secretion of primed cells. Human peripheral blood was activated with PHA and then restimulated in the presence of LPS in control medium or I_E water medium. The results are shown in Figure 1 and are representative for TNF-α. Clearly, in the absence of prior activation, I_E water stimulates TNF-α secretion as expected. However, following priming, the response obtained in I_E water is significantly and synergistically higher than the response obtained in control water.

These findings show clearly that I_E water preparations can potentiate the recall response and act in a synergistic fashion.

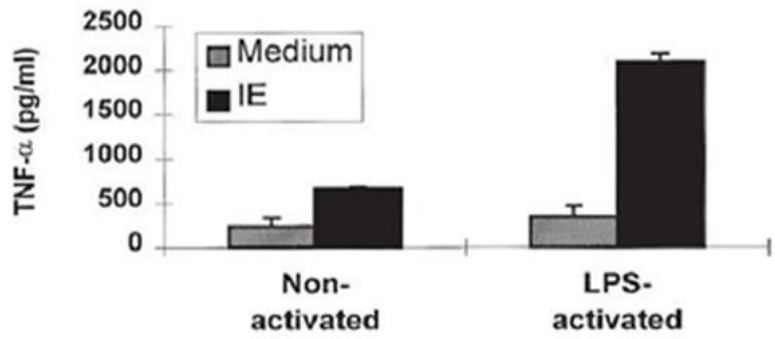


Figure 1 ***Synergistic response to PHA in activated blood cells****.*

Human peripheral blood was prepared as described in the legend of Table 1. The cells were cultured with or without LPS (10 ng/ml) for 18 h. The cultures were replaced with fresh medium in the presence of PHA (5 µg/ml) and incubated for 48 h. Cell-free supernatants were harvested and stored for assessment of cytokines.

3.2 Effect of I_E Water on Peripheral Blood Monocytes (PBM)

3.2.1. Spontaneous Induction

In the previous sections, we examined the effect of I_E water preparations on whole blood which mimics the in vivo system. However, we wished to investigate the effect of I_E water preparation on separated subsets of human blood leukocytes in order to determine if there is a preferential induction of cytokines by the subsets and if there is also a selective triggering of certain cytokines over others. We examined the cytokine secretion of purified human peripheral blood monocytes (PBM).

Table 5 shows that I_E water significantly stimulated the secretion of TNF-α, IL-6 and IL-12 by PBM as compared to control water preparations. The induction of cytokines is at the level of gene induction and transcription as shown in Figure 2.

We performed RT-PCR to determine the regulation of TNF-α synthesis. Clearly, control water has no detectable mRNA for TNF-α while I_E water induced significant levels of mRNA and was comparable to control LPS- induced TNF-α. These findings demonstrate that I_E water stimulates TNF-α secretion by gene induction.

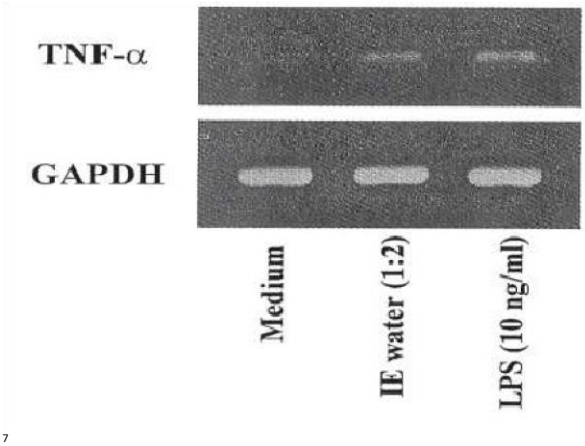
Table 5. I_E water-induced cytokine production by human peripheral blood monocytes.

Water Preparation	Cytokine (pg/ml)		
	TNF-α	IL-6	IL-12
Laboratory	1359	168	11
ATG Control	979	169	6
I_E #1	16740	2579	76
I_E #2	22851	2534	215

The purified peripheral blood monocytes were prepared as described in Materials and Methods. The cultures were prepared as described in the legend of Table 1.

3.2.2 Kinetics of Secretion

We examined the time kinetics of TNF-α secretion by PBM cultured in I_E water and compared it to a control medium preparation. Figure 3 is a representative experiment which demonstrates that TNF secretion is detected early after culture in I_E water (<4 h) and increases as a function of incubation time. It reaches a plateau at 1 day and depending on the subjects, either it remains at plateau level for several days as shown in Figure 3 or declines (not shown). PBM in control medium show some spontaneous induction at 8 h and plateaus thereafter.



[7]

Figure 2 Gene induction of cytokines by Ig (PCR). RT-PCR was done as described previously using human peripheral blood monocytes.

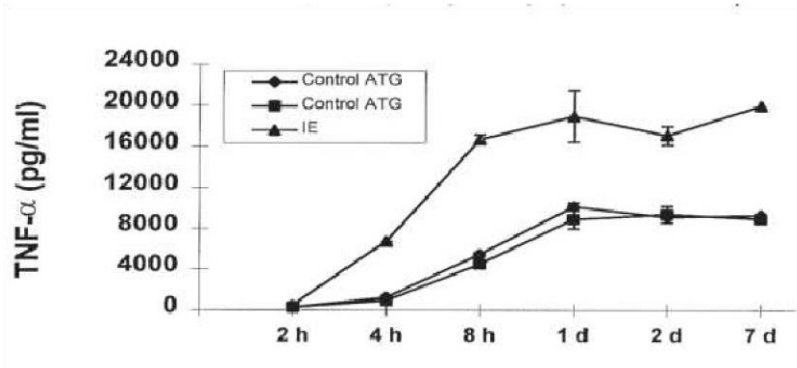


Figure 3. Kinetics of TNF-α secretion by human monocytes. This experiment was done according to the procedure described in the legend of Table 6.

Furthermore, the kinetics of secretion of other cytokines (IL-6, IL- 10, IL- 12) is shown in Table 6. When compared to LPS, we found that for IL-10, the I_E water stimulation was significant but less effective than LPS. In contrast, for IL-12, 1E water stimulated higher levels than LPS and increased from day 1 to day 2 (Figure 4).

Table 6 Time kinetics of I_E -induced cytokine secretion by monocytes.

Time	TNF-α		IL-6		IL-10		IL-12	
	Control	I_E	Control	I_E	Control	I_E	Control	I_E
2 h	668	5305	57	82	4	4	13	14
4 h	2220	19274	188	1997	4	4	14	14
8 h	2245	24999	1689	2835	4	13	18	50
24 h	610	9465	2372	2775	4	190	34	139
48 h	241	992	3125	3125	5	109	37	155
7 days	4072	819	2741	2741	5	41	41	78

In this experiment, 106/ml purified monocytes were incubated with IE medium 50% or control medium. At different time points, cell free supernatants were harvested and stored at 70oC, for future use, for cytokine determination.

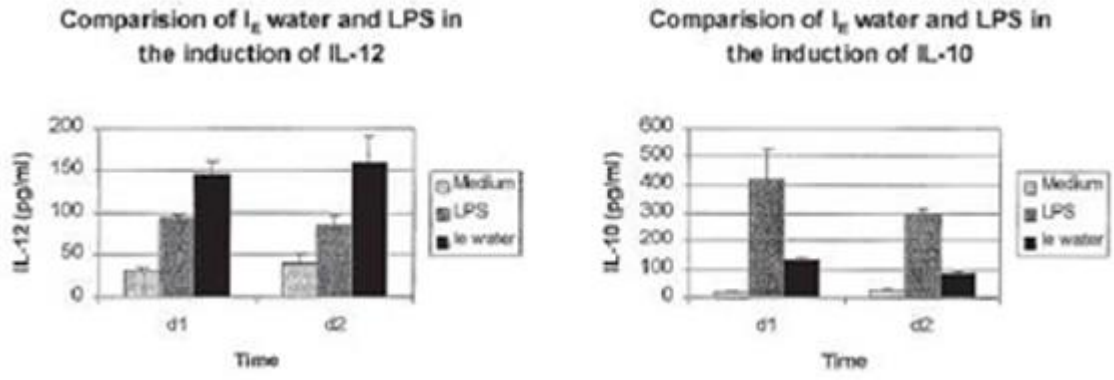


Figure 4. This experiment was done according to the legend of table 3.

The cultures were harvested at d1 and d2 and cell-free supernatants stored for the assessment of cytokines

3.3 Effect of I_E Water Preparations in Non-Adherent Peripheral Blood Leukocytes

We examined the effect of I_E water on cytokine secretion by non-adherent lymphocytes (T, B, NK). The findings in Table 7 demonstrate that significant levels of TNF-α and IL- 12 were stimulated by I_E water compared to control. These findings demonstrate that I_E water can also stimulate lymphocytes in addition to monocytes as described in 3.2 above.

Table 7 Effects of I_E water on cytokine secretion by nonadherent PBMC.

Water Preparation	Cytokine (pg/ml)		
	TNF-α	IL-6	IL-12
ATG Control	13,000	2016	120
I_E #1	38,765	2119	720

Peripheral blood mononuclear cells were isolated by Ficoll Hypaque and adherent cells were depleted by adherence to plastic plates. Two million cells/ml in different media were incubated for 48 h and cell-free supernatants collected for assessment of cytokines.

3.4 Absence of Endotoxin in I_E Water Preparations

Since I_E water preparations stimulated several cytokines by peripheral blood, we examined whether contaminating levels of endotoxins, powerful mitogens, might be responsible for the I_E mediated effects. We used an LPS-specific inhibitor, polymixin B, which inhibits the potent LPS mitogen. The findings in Figure 5 demonstrate that while polymixin B inhibits LPS-mediated stimulation of TNF-α, polymixin B has no effect, albeit enhancing effects on I_E induced TNF-α secretion. These findings clearly indicate that the I_E -mediated cytokine induction is not due to endotoxin contamination in the I_E water preparations.

4 Discussion

The findings in these studies suggest that human peripheral blood-derived leukocytes cultured in medium prepared with I_E crystal water undergo a state of activation resulting in cytokine production. Several cytokines are secreted albeit at various levels. Furthermore, certain cytokines are preferentially secreted over others. In addition, I_E water preparations act synergistically with T and B mitogenic stimulation. Noteworthy, the induction of cytokine secretion is rapid and is detected at less than 4 h following culture and increases progressively and reaches a plateau which is maintained for several days.

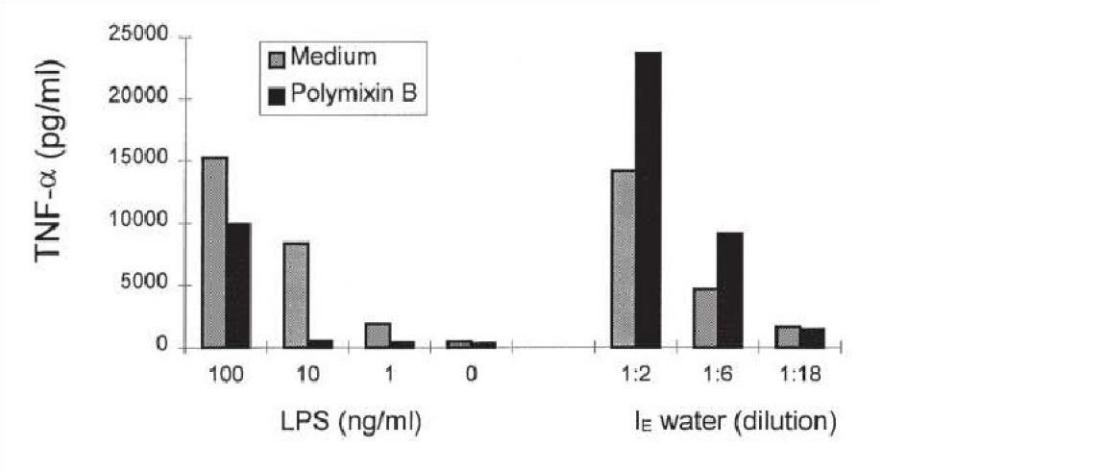


Figure 5. Absence of endotoxin in IE preparations.

The cell preparations were incubated with LPS (10 ng/ml) in the presence or absence of polymixin B (10 µg/ml) and incubated for 18 h. Cell-free supernatants were harvested and stored for cytokine measurement.

The stimulation of cytokines by I_E water is mediated through gene induction. Altogether, these findings demonstrate for the first time that water containing I_E crystals affect human blood leukocytes in a manner that mimics mitogenic or antigenic stimulation.

The I_E water preparation used in these studies did not contain detectable endotoxin contaminants like LPS that may be in part responsible for the induction of cytokines. This was demonstrated by the use of specific endotoxin inhibitors. Further, water preparations

made under similar conditions, with the exception of induction of I_E crystals, did not have any detectable activity.

The stimulation of significant levels of cytokines by I_E and potentiation with antigenic stimuli suggest an important regulatory role in the immune response. The nature and level of cytokines produced will influence a particular immune response.

Mossmann et al.[3] reported an intriguing and conceptual revolution in immunology by dividing T helper (TH) cells into two populations (TH 1 and TH2) with contrasting and cross-regulating cytokine profiles. This new paradigm was enthusiastically endorsed in many areas of immunology and infectious diseases.

The TH 1 response evolves around the production of IFN-γ and the subsequent activation of macrophages. These features of cell-mediated immunity are certainly important for the resolution of intracellular infections[8,9].

The paradigm that TH2 cells protect against extracellular parasites arise in part from the established role of antibodies in the control of many extracellular pathogens and the role that the TH2-type cytokines IL-4, IL-S and IL-6 play in the generation of an effective antibody response [10,11]. In addition, autoimmune diseases appear to be in general TH1 - mediated [12]. For example, experimental work in insulin-dependent diabetes mellitus and experimental allergic encephalitis are TH1-mediated immune manifestations.

Likewise, immune defense mechanisms against cancer are, in some instances, mediated by TH1-type responses.

Further, transplantation studies show that graft rejection in TH1-mediated and graft acceptance is TH2-mediated [13].

To date, it is more appropriate to define the combination of cytokines and effector cells required for a successful immune response than to attempt to clarify protective immunity as TH1-type or TH2-type. Other cells than T cells (example NK and monocytes) produce cytokines that may achieve a TH 1 or TH 2 effect [14].

Clearly, the findings of the present study are provocative as they demand understanding of the underlying molecular mechanisms by which I_E crystals initiate the activation machinery in the cells to secrete cytokines. Clearly, one of the most important questions to answer is the initial trigger between water and cells.

It is possible that I_E water introduces changes in cell membrane potential that can result in triggering receptor-like signaling pathways. It is also possible that I_E water bypasses membrane effects and activates intracellularly enzymatic activity that initiates the cascade of activation signaling resulting in gene transcription and translation of cytokine

gene products. Other yet unknown effects can also be induced by I_E water. These and other studies are urgently needed.

The present findings with I_E crystal preparations were all performed with one particular type of cluster. It is possible that different cluster preparations will have different effects and will activate cytokines differentially. Schematically shown in Figure 6 are the possible outcomes of different I_E crystal preparations (crystals A-D).

Briefly, preparation I_EA may selectively regulate cytokines involved in cell-mediated immunity by potentiating TH 1 cytokines.

Preparation I_EB may be potentiating TH2 cytokines and regulates antibody responses.

Preparations I_EC may be potentiating cytokines that regulate phagocytosis of pathogens.

Preparation I_ED may regulate autoimmune responses and allergies.

These and other preparations may be, in principle, customized by the preparations of different crystals or mixture of crystals. Clearly, the implication of these various water preparations in vitro and in vivo in the regulation of immune responses must be investigated.

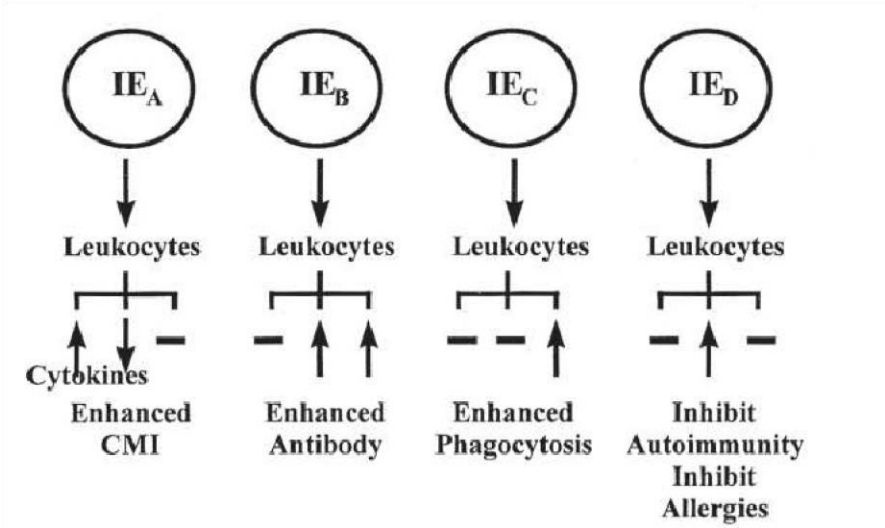


Figure 5 Cytokine regulation by IE. Schematic diagram showing regulation of cytokine production by leukocytes. ↑represents upregulation; ↓represents downregulation; -represents no effect.

In conclusion, these studies provide the initial phase of our long term objectives by exploring the biological effect of I_E water preparations on mammalian tissues and immunity. Furthermore, the principles of I_E crystal preparations must be examined as they may also apply in homeopathic remedies.

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