

INVERSE RELATIONSHIP BETWEEN NET ELECTRIC CHARGE
ON THE ANTIGEN AND THAT ON THE SENSITIZED
CELL IN CELLULAR IMMUNE RESPONSE: DEMONSTRATION
WITH BASIC ENCEPHALITOGEN OF THE BRAIN*

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An inverse relationship has been demonstrated between the net electrical charge of immunogens and the charge of the antibodies elicited by them (1-3). IgG antibodies to natural and synthetic negatively charged immunogens were found in the first, more basic, fraction of immunoglobulin eluted from diethylaminoethyl Sephadex A-50. In contrast, the antibodies elicited by basic immunogens appeared in the second, more acidic, eluted fraction (1-5). This observation has been extended to include antihapten antibody responses generated by groups such as 2,4-dinitrophenyl and a tetrapeptide of D-alanine, attached to positively and negatively charged carriers (1, 2, 6). These hapten-specific antibodies differed in net electrical charge, but were indistinguishable with respect to their specificity and affinity (6). It has been previously established that the inverse net charge phenomenon has a cellular basis (7, 8). Thus, the immune response potential of mouse spleen to the 2,4-dinitrophenyl hapten attached to a negatively charged synthetic polypeptide carrier was reduced by cell fractionation over negatively charged glass bead columns, whereas the response to the same hapten on a positively charged carrier was unaffected (7). Furthermore, spleen cells fractionated over positively charged poly-L-lysine-coated glass bead columns showed reduced response potential to the dinitrophenyl hapten on the positively charged carrier only.

It was established that the cell population relevant for the charge properties of immunogens was of thymus and not of marrow origin, by experiments in which thymocytes and bone marrow cells were selectively passed over positively or negatively charged columns and mixed with unfractionated cells of the complementary type (8). Thus, thymocytes fractionated over negatively charged columns and mixed with unfractionated marrow cells exhibited reduced antibody response to the hapten on the negative carrier but normal responses to hapten

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on the positive carrier. The opposite result was obtained when thymocytes were passed over positively charged columns. No effect on the antihapten antibody formation was detected by filtration of bone marrow cells over columns of either charge. These findings indicated that it is possible to distinguish between thymocytes on the basis of their capacity to react with more acidic or more basic surfaces and that a population of thymus-derived cells may recognize immunogens on the basis of their overall electrical charge.

In the present study we attempted to establish whether the inverse charge effect can be extended to include cell-mediated immune responses. Basic encephalitogenic protein (BE)¹ of the central nervous tissue seemed a particularly suitable antigen for such a study since it is a highly basic molecule that induces an autoimmune syndrome which is easily detected. This autoimmune neurological disease, called experimental allergic encephalomyelitis (EAE), involves clinical manifestations of weight loss, incontinence, and paralysis of the hind limbs, as well as histological lesions in the central nervous system. EAE is generally considered to involve cell-mediated immunopathological mechanisms rather than direct effects to humoral antibody (9). In this study we have verified that the inverse net charge relationship prevails also in phenomena involving purely cellular immunity.

Materials and Methods

BE. BE was purified from bovine spinal cord by delipidation, acid extraction, and column chromatography on sulphoethyl-Sephadex as described previously by Hirshfeld et al. (10).

Antigens. Hen egg-white lysozyme (twice crystallized) was obtained from Worthington Biochemical Corp., Freehold, N. J. Complete Freund's adjuvant (CFA), and phytohemagglutinin (PHA)-M, were obtained from Difco Laboratories, Detroit, Mich. *Mycobacterium tuberculosis* H₃₇R_v was a gift from Dr. O. Moscovici of the Zamenhof Central Laboratories of Kupat Holim, Tel Aviv, Israel.

Cell Culture Media. Minimum essential medium for suspension (MEM-S) with added nonessential amino acids, and glutamine, penicillin, and streptomycin solutions were obtained from Microbiological Associates, Jerusalem, Israel. Normal guinea pig serum was obtained from Grand Island Biological Corp., N. Y.

Animals. For all transfer experiments strain 13 guinea pigs, weighing 450 g or more, were used as donors, and syngeneic animals weighing 200 g or less were used as recipients.

Preparation of Negatively and Positively Charged Columns. Columns of uncoated glass beads (Superbrite, 100-5,005; Minnesota Mining & Manufacturing Co., St. Paul, Minn.) were prepared according to the method described by Sela et al. (7). Poly-L-lysine-coated glass beads were prepared as described by Karniely et al. (8).

The columns consisted of glass pipettes, 22.5 cm long x 0.86 cm diameter. Fine wire mesh was placed in the bottom of each pipette to retain the beads. The void vol of the columns packed with beads was 4 ml.

Lymph Node Cell (LNC) Suspensions. Lymph nodes were excised, trimmed of fat, and teased apart under sterile conditions in MEM-S supplemented with 13% inactivated normal guinea pig serum. Cells were washed in the same medium and resuspended at the desired concentration.

Cell Fractionation on Columns. LNC suspensions (10⁸ cells/ml) in MEM-S containing 13% homologous serum were loaded on the columns in a total vol of 2-3 ml. The cells were passed through the columns at 4°C. The unbound cells were eluted with cold medium, at a flow rate of 3-4

¹ *Abbreviations used in this paper:* BE, basic encephalitogenic protein; CFA, complete Freund's adjuvant; EAE, experimental allergic encephalomyelitis; LNC, lymph node cells; MEM-S, minimal essential medium for suspension; PHA, phytohemagglutinin.

ml/min. The cell suspensions were centrifuged and resuspended at the desired cell concentration for either cell transfer or lymphocyte transformation experiments.

Cell Transfers. For passive transfer of EAE, donor strain 13 guinea pigs were sensitized with an emulsion of whole guinea pig spinal cord homogenate in CFA (0.5 ml of emulsion containing 25% spinal cord by wet weight and 2.5 mg dry weight of *M. tuberculosis* H₃₇R_v) in the four foot pads and in the nuchal area. Lymphocyte suspensions for cell transfers were prepared from the draining lymph nodes of the donor animals 5–6 days after immunization. The suspensions (2 ml) were injected into recipient animals intraperitoneally at a concentration of 5×10^8 cells/ml. The entire procedure was conducted at 4°C.

Lymphocyte Transformation. These experiments were conducted as described previously (11). The animals were sensitized with 20 µg of purified BE in CFA in all four foot pads. Draining lymph nodes were excised 12–20 days after sensitization. The lymphocytes $8-10 \times 10^6$ cells/culture were incubated for 24 h with varying concentrations of the test antigens, followed by 24 h incubation with 0.1 µCi of [2-¹⁴C]thymidine (New England Nuclear, Boston, Mass.). The cells were then filtered on Whatman glass fiber paper GF/C, and washed successively with saline, trichloroacetic acid (5%), and absolute ethanol. The filters were dried and the radioactivity measured in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The results are expressed as stimulation indices, namely the ratio of the radioactivity (counts per minute) in tubes containing antigen, to counts per minute of antigen-free tubes (control), and they represent an average of values obtained in duplicate cultures.

TABLE I
Yield and Viability of Cells Filtered Over Charged Columns

	Filtered over glass beads	Filtered over poly-L-lysine-coated glass beads
Cells loaded*	5.5×10^8	9.5×10^8
Cells eluted	2.8×10^8	1.6×10^8
Cells retained	49%	83%
Viability (trypan blue exclusion)	90%	90%

* Each column was loaded with $1.0-1.5 \times 10^8$ cells in 1–2 ml of medium containing 13% normal serum.

Results

Cell Filtration Over Glass Beads and Poly-L-Lysine-Coated Glass Beads. Strain 13 guinea pigs were sensitized for cell transfer experiments with whole nervous tissue homogenates or, for lymphocyte transformation studies, with purified BE. LNC suspensions were prepared and fractionated over glass bead columns (which are negative) or poly-L-lysine-coated glass bead columns (which are positive).

The fractionation system was characterized in terms of cells retained vs. cells eluted and in terms of cell viability, as summarized in Table I. We found that 50% of the cells were eluted from the glass bead columns which were equilibrated in medium containing 13% normal guinea pig serum to avoid retention of the majority of cells. These cells were as viable as unfractionated LNC suspensions, i.e. 90% viability, as determined by exclusion of trypan blue. From the columns of poly-L-lysine-coated glass beads only 20% or less of the cells were eluted under identical conditions, again, with full viability.

TABLE II
Lymphocyte Transformation of BE-Sensitized Cells Fractionated over Charged Columns

Antigen	Concentration	Cells filtered over glass beads		Cells filtered over poly-L-lysine-coated glass beads		Unfiltered cells	
		Δ cpm	SI*	Δ cpm	SI	Δ cpm	SI
	<i>$\mu\text{g/culture}$</i>						
BE	10	2,392	2.2	—	0.9	1,181	2.3
	50	5,130	3.1	—	0.9	1,672	2.9
	100	8,509	4.5	530	1.2	2,900	4.3
	200	5,603	3.3	—	0.8	1,935	3.2
PHA	100	47,966	20.3	40,211	17.0	22,428	26.5

* The values represent the stimulation indices (SI) of incorporation of [^{14}C]thymidine by the cells.

Lymphocyte Transformation of Fractionated Cell Populations. The fractionated cell populations were compared to the original LNC suspensions for their ability to respond to the BE in vitro. Lymphocyte transformation in the presence of BE was measured in terms of incorporation of radioactive thymidine relative to unstimulated controls. The results of these experiments are shown in Table II. Unfractionated LNC gave significant responses to BE in vitro, the optimal concentration falling in the range of 100 $\mu\text{g/culture}$. The cells eluted from positive columns (poly-L-lysine-coated glass beads) are essentially unresponsive to BE, whereas the response to a nonspecific mitogen such as PHA is unimpaired. In other words, passage on positive columns results in depletion of the cells capable of responding to the positive sensitizing antigen. In contrast, cells eluted from negative columns (glass beads) are virtually indistinguishable from the original cell population in terms of the stimulation indices obtained. When the response is calculated on the basis of the increment of thymidine incorporated (Δ counts per minute) there appears to be an elevated level of response in the cell suspension eluted from the negative columns.

Passive Transfer of EAE with Fractionated Cell Populations. The fractionated cell populations were compared to the whole cell population for their ability to transfer EAE to unsensitized syngeneic recipients.

Whole LNC suspensions successfully transferred clinical EAE to 8/10 recipients (Table III). In the adoptive disease the clinical manifestations did not involve actual paralysis. Definite signs of paresis were considered clinically positive. This state was usually observed 7–10 days after injection of LNC and was presaged by a considerable loss of weight. All the recipients of the unfractionated LNC exhibited severe histological lesions in their brain.

Cells eluted from negative columns produced less overt clinical symptoms, which primarily included loss of weight and loss of righting reflex. The incidence of these clinical signs was not lower than in the controls (7/7). Histologically the incidence of lesions was also as in the controls, however the degree of damage

TABLE III
Passive Transfer of EAE by Cells Filtered Over Charged Columns

Cell population	Donor/ recipient*	Clinical incidence		Histological changes	
		Loss of weight	Paresis	Incidence	Degree
Unfiltered	2-3/1	10/10	8/10	10/10	++,+++
Filtered over glass bead columns	5-6/1	7/7	0/7	7/7	+,++
Filtered over poly-L-ly- sine-coated glass bead columns	15-20/1	0/4	0/4	0/4	—

* Each recipient received 10^9 cells intraperitoneally in 1.5-2 ml.

was lower (Table III). On the other hand, cells eluted from positive columns are apparently devoid of the capacity to induce EAE as evaluated either by clinical or histological criteria. Only a limited number of animals was tested due to the large ratio of donors to recipients necessary in order to obtain sufficient cells (10^9) to transfer to each recipient. However, not a single animal of this group developed any signs of EAE (0/4).

Discussion

In the present study we have demonstrated that the capacity to respond to the positively charged BE is confined to the fraction of more negatively charged cells. This inverse charge effect was demonstrated both *in vitro*, as determined by the lymphocyte transformation responses in the presence of BE, and *in vivo*, in terms of the capacity to transmit EAE to unimmunized syngeneic animals.

A population of immunocompetent cells that can discriminate between immunogens as a function of their charge properties (in addition to or instead of recognition based on specificity) has been shown to undergo fractionation on charged columns. This inverse net charge phenomenon has a cellular basis for both positively and negatively charged immunogens as demonstrated by cell separation techniques over columns of opposite charge (7, 8). Mouse spleen cells eluted from glass bead columns or poly-L-lysine-coated glass bead columns comprised 35 or 20% of the whole cell suspension, respectively. In the present study guinea pig LNC were found to be retained on positive columns to the same extent as mouse spleen cells though the yield from negative columns was consistently higher (Table I).

A specific *in vitro* response to BE was obtained only in the population of cells eluted from the negative columns. When the results are expressed in terms of stimulation indices, the response of this population is virtually identical to that of the whole LNC cultures. When expressed in terms of Δ counts per minute, the response of the fractionated population is greater than that of the entire population. This may reflect an actual enrichment of the relevant cells which undergo transformation in the presence of BE. However, the background level of incorporation is elevated in the fractionated populations as well, probably due to some nonspecific stimulation by passage through the column. In previous experi-

ments the functionality of fractionated mouse spleen cells was assayed in terms of antibody production *in vivo*, and no enrichment was found as regards the proportion of responding animals (7).

In our system the *in vivo* functionality of eluted cells was assayed by their capacity to induce signs of EAE. The cells eluted from glass bead columns transferred both histological and mild clinical symptoms to syngeneic recipients (Table III). The functionality of these positive cells was somewhat diminished in comparison to the original whole cell suspension, and this may reflect the loss of some labile factor vital to the successful passive transfer of EAE.

The responsiveness to BE is completely absent from the fraction of positive cells in both the *in vitro* assay and the passive transfer of EAE (Tables II and III). The major encephalitogenic determinant of the BE molecule in guinea pigs resides in the immediate vicinity of the sole tryptophan residue (12, 13). This peptide is in itself neutral at physiological pH, nevertheless it is in a highly basic environment. This situation may reflect the same sort of effect as that of a hapten on a charged carrier (6). For such systems it has been suggested that over and above the selection of the antibody-combining site by the antigenic determinant, the carrier portion of the immunogen largely determines the chemical nature of the antibody formed (1). On the level of cellular responses the same phenomenon may serve as a basis to explain our findings.

In the present study we have extended this inverse charge effect to include a cell-mediated immune response of the delayed hypersensitivity type, namely the response to BE and the passive transfer of EAE. Whereas the transfer of EAE by LNC does not in itself rule out the contribution of B cells in the pathogenic mechanisms, indirect evidence tends to implicate primarily T cells (14-16). This would also gain credence from the previous finding that thymocytes rather than bone marrow cells account for the charge effect of whole spleen populations (8), though in that study helper cell function was assayed rather than a cellular immune response by itself. Recently, it has been demonstrated that there is a dissociation between the T-helper-cell function and delayed hypersensitivity (17, 18). We have now established the inverse charge effect for delayed-type hypersensitivity.

Summary

An inverse relationship exists between the net electrical charge of immunogens and the antibodies elicited (1). The cellular basis of the net charge phenomenon has been established for both positively and negatively charged immunogens, by cell separation techniques over columns of opposite charge (7, 8). To establish whether this phenomenon can be extended to include cell-mediated immunity, the response to basic encephalitogenic protein (BE) which induces experimental allergic encephalomyelitis (EAE) was now investigated. Lymph node cells from sensitized strain 13 guinea pigs were fractionated over positively and negatively charged columns and compared to unfractionated cell populations in two assay systems: (a) *in vitro* response to BE in terms of lymphocyte transformation and (b) the passive transfer of EAE to unsensitized syngeneic recipients. The response was found to be confined to the fraction of cells eluted from glass bead columns, namely, the more negative cells. Cells eluted from

poly-L-lysine-coated glass bead columns (i.e., positive cells) were devoid of the capacity to respond to this antigen either *in vivo* or *in vitro*.

It was previously established that thymocytes rather than bone marrow cells account for the inverse charge phenomenon as assayed by T-helper-cell function in *in vivo* antibody production (8). We have now extended the inverse charge effect to include cell-mediated immune response of the delayed hypersensitivity type.

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