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Cellular Aspects of the Inverse Relationship between the Net Charge of Immunogens and of Antibodies Elicited

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Abstract. The results reported establish a cellular basis for the inverse relationship between the net electrical charge of immunogens and of the antibodies provoked by them. Glass bead columns were used to reduce the number of immunocompetent spleen cells preferentially reactive with more acidic immunogens. After a single immunization, titers of antibodies to an acidic dinitrophenylated copolymer of tyrosine, glutamic acid, and lysine (DNP-901) elicited in recipient mice by filtered spleen cells were significantly lower than those generated by unfiltered cells. After secondary stimulation, the major portion of the antibodies provoked by the acidic antigen was found in the more acidic fraction eluted from DEAE-Sephadex, in contrast to the more basic antibodies, of the same specificity, generated by unfiltered spleen cells. Results obtained by transplanting a limiting number of spleen cells indicate a depletion in the number of precursor cells reactive with dinitrophenyl on DNP-901 after glass bead chromatography, whereas there was no change in the response to dinitrophenyl on a basic copolymer, DNP-912, containing the same amino acids in different molar ratios.

The inverse relationship between the net electric charge of immunogenic macromolecules and the net charge of antibodies provoked by them has been demonstrated for antibodies produced in rabbits[1,2], mice[3], goats[4], and humans[5], and is valid for antibodies of IgG[6], IgM[7], and, probably, IgE[5] class. Thus, e.g., antibodies to the acidic diphtheria toxoid and to negatively-charged synthetic copolymers of amino acids appear mainly under the first peak upon fractionation on DEAE-Sephadex[1,8], whereas antibodies to the basic lysozyme and positively-charged amino acid copolymers appear predominantly, or exclusively, under the second peak[1,3].

Haptens such as the dinitrophenyl group[1,2] or a peptide of L-alanine (unpublished data of Licht, A., B. Schechter, I. Schechter, and M. Sela) will lead to antibodies fractionating under the first or the second DEAE-Sephadex peak, depending on whether they are attached to a negatively or positively charged carrier. The anti-hapten antibodies produced upon immunization with such conjugates differ in their net electric charge, but not in their specificity or affinity, as measured by means of small molecules related chemically to the original haptens. The inverse correlation described depends on the net electric charge of the intact immunogen, and not on the net charge within a limited area around the anti-

genic determinant[9]. The difference between the two antibody fractions is reflected primarily in the light[10], not the heavy[11], chains.

We have now investigated the cellular nature of this phenomenon with an experimental procedure based on the fractionation of mouse spleen cells on glass beads, and their transfer into irradiated mice, followed by administration of the antigen. The results indicate that the inverse relationship between the net charge of immunogens and of antibodies they provoke is due to a preferential selection of cells generating the antibody production.

Materials and Methods. The acidic synthetic copolymer DNP-901 has been previously described[1] under the name 901(T,G,L). It is dinitrophenyl poly $Glu^{83}Lys^{12}Tyr^{5}$ (all L-amino acid residues) and contains 1.65% dinitrophenyl groups. DNP-912 is a basic copolymer, Dnp-poly $Glu^{19}Lys^{79}Tyr^{6}$, containing 2.2% Dnp groups. These copolymers and egg white lysozyme (Mann) were used for immunization. Dnp-rabbit serum albumin containing 8% Dnp groups was used as the cross-reacting antigen for titration of Dnp-specific antibodies.

(Balb/c × C57Bl/6) F_1 female mice, 9–11 weeks of age, obtained from the experimental animal unit of our institute, were used throughout the experiments. Spleen cell suspensions were prepared from donors and adjusted to 5×10^7 cells per ml. A portion of the cells was passed through columns of uncoated glass beads (Superbrite, 100–5005, 3M Co., St. Paul, Minn.) prepared according to Wigzell and Andersson.[12] 5×10^7 cells were passed through each 22.5 × 0.86 cm column, and the cells were eluted with Eagle's medium (Grand Island Biological Co.). Approximately half of the total cell number was recovered in the effluent. Of the cells eluted from the original column, 94% was recovered after filtration on a fresh column. A second portion of cells was not passed over glass beads. The filtered cells as well as those not passed over glass beads were diluted to 2×10^6, 4×10^6, and 8×10^6/ml, and inoculated into syngeneic recipients exposed to 750 R of x-radiation[13]. 24 hr later, each group of recipients was inoculated intraperitoneally with 10 μg of one of the above immunogens in complete Freund's adjuvant (Difco Laboratories). 14 days later (at the time of peak response), the mice were bled and their sera were titered individually for anti-Dnp antibodies by passive microhemagglutination[14] with tanned sheep erythrocytes coated with Dnp-rabbit serum albumin and for anti-lysozyme antibodies with lysozyme-coated erythrocytes. The groups of mice inoculated with 8×10^6 spleen cells were again stimulated with the immunogens immediately after bleeding, and were bled again and their sera titered 10 days later.

Chromatography on DEAE-Sephadex A-50 medium (Pharmacia, Uppsala) columns, 16 × 1.5 cm, was performed using 2-ml aliquots of mouse sera as described previously.[1,3]

Results. In a number of animal species a correlation has been demonstrated between the net charge of immunogens and that of the antibodies elicited by them.[1–5] If a population of potentially immunocompetent cells exists that can select immunogens on the basis of net antigenic charge, in addition to recognition based on determinant specificity, then more positively charged immunocompetent precursor cells would be expected to react preferentially with more acidic immunogens. Should the above assumption be correct, one would predict that more acidic immunocompetent cells will be more readily eluted from columns of glass beads, since glass is acidic, whereas more basic cells should adhere to such surfaces. Consequently, cells collected after glass-bead filtration should be preferentially stimulated by the more positively charged immunogen.

Irradiated (Balb/c × C57Bl/6) F_1 mice were divided into two groups, and inoculated either with 8×10^6 spleen cells that had been filtered through columns of glass beads or with an equal number of unfiltered spleen cells. Each group of

recipients was immunized with the acidic DNP-901 or with the basic protein, egg white lysozyme. The mice were bled 14 days later, before the second stimulation with the immunogens. The sera were titered for anti-Dnp and for anti-lysozyme antibodies.

After the immunization with lysozyme, antibody titers for the groups of mice inoculated with filtered and unfiltered cells were very low (1:4–1:8). Reimmunization yielded titers (1:16–1:32) that were similar for both groups. After the first immunization with DNP-901, all 11 recipients that received unfiltered cells responded with hemagglutination titers ranging from 1:32 to 1:64, whereas 13 of 14 recipients inoculated with filtered cells responded with significantly lower titers, ranging from 1:8 to 1:16 (one mouse had no detectable response). After restimulation with DNP-901 the titers from recipients of filtered and unfiltered cells were indistinguishable (1:64–1:128).

Although after the immunization, there was a 4-fold difference in anti-Dnp titers between recipients of filtered and unfiltered cells, no significant difference was detected in the antibody titers for acidic and basic immunogens after restimulation. Therefore, it was of interest to determine the charge of the antibody populations produced. Pooled antisera from the groups of animals described above, as well as from normal, unirradiated mice inoculated with lysozyme or DNP-901, were chromatographed on DEAE-Sephadex columns. The antibody titers in the two eluted fractions were determined. The ratios of antibody activity in the second to the first fraction were calculated, and are shown in Table 1. The results obtained with sera from normal, unirradiated mice show

TABLE 1. *Distribution of anti-Dnp and anti-lysozyme antibodies, after DEAE-Sephadex chromatography, in sera of normal mice, and of irradiated mice inoculated with glass-bead-filtered or unfiltered spleen cells.*

Mice immunized*	Ratio of antibodies in second to first fraction (immunized with lysozyme)	Ratio of antibodies in second to first fraction (immunized with DNP-901)
Not x-irradiated	13.2	0.3
X-irradiated with unfiltered cells	6.2	0.7
X-irradiated with filtered cells	6.6	2.1

* Pooled sera from 11–15 mice were used for each analysis.

the expected distribution of antibodies in the two fractions eluted from DEAE-Sephadex columns.[1] The second fraction contained most of the antibodies to the basic lysozyme, whereas the antibodies to the acidic DNP-901 were found predominantly in the first fraction. The ratios of the second fraction to the first fraction were 13.2 and 0.3, respectively. The distribution of antibodies in the sera of irradiated mice inoculated with 8×10^6 unfiltered cells was similar to that in the sera of unirradiated animals, although the titers were lower. In contrast, the majority of antibodies elicited by DNP-901 in sera from recipients given 8×10^6 glass-bead-chromatographed spleen cells was detected in the second fraction. On the other hand, no difference was observed in the ratio of the two fractions for antibodies to the basic lysozyme, independently of whether the recipient mice had received filtered or unfiltered cells.

In order to verify the cellular basis for correlation between net charge of antigens and that of the antibodies elicited, the experimental design was modified by reducing the number of spleen cells transferred into irradiated mice to a point where not all recipients received a functional immunocompetent unit of response.[13,15] In these experiments the basic copolymer DNP-912, which gives in mice an anti-Dnp response resembling that of the acidic DNP-901, was used instead of lysozyme. Irradiated recipients were divided into groups and inoculated with 2×10^6 or 4×10^6 filtered or unfiltered spleen cells. Each group was then immunized with DNP-901 or DNP-912. 14 days later, recipient sera were collected and individually assayed for anti-Dnp antibodies. The results, expressed as the fraction and percentage of positive responses detected in recipient sera, are shown in Table 2. Glass-bead filtration had no appreciable effect

TABLE 2. *Percentage of positive sera in irradiated mice fourteen days after inoculation of glass-bead-filtered or unfiltered spleen cells and immunization with dinitrophenylated acidic or basic copolymers.*

Cells passed over glass beads	Immunizing antigen*	Fraction of positive sera (4×10^6 cells†)	Percentage of positive sera (4×10^6 cells†)	Fraction of positive sera (2×10^6 cells†)	Percentage of positive sera (2×10^6 cells†)
No	Acidic‡	11/15	73.3	7/11	63.6
Yes		5/14	35.7	4/14	28.6
No	Basic§	12/18	66.7	8/17	47.1
Yes		10/15	66.7	3/5	60.0

* 10 μg of immunogen was inoculated intraperitoneally in complete Freund's adjuvant after cell transfer.
† Number of spleen cells inoculated into irradiated recipients.
‡ DNP-901.
§ DNP-912.

on the frequency of responses to the basic DNP-912, whereas a significant reduction was observed in the number of positive sera of recipients inoculated with filtered cells and the acidic DNP-901. These results suggest a depletion in the number of relevant precursor cells reactive with Dnp groups on DNP-901 after glass-bead filtration. On the other hand, no marked change in the number of limiting precursor cells sensitive to Dnp groups on the basic polymer was observed.

Discussion. Previous work has demonstrated unequivocally an inverse correlation between the net electrical charge of the intact antigenic macromolecule and that of the antibodies provoked by it. It was proposed that the nature of such antigenic control of the type of antibodies synthesized involves a process of antigenic selection at the cellular or subcellular level.[1] Therefore, it was of fundamental importance to determine whether this phenomenon has a cellular basis. In fact, from binding affinity studies of Dnp-specific antibodies, it has been suggested that charged antigens preferentially select populations of cells synthesizing antibodies of opposite charge.[2]

The objective of the present study was to selectively bind the more basic cells, that were reactive with acidic antigen, by filtration on columns of glass beads. Such a procedure does not involve cell enrichment due to antigenic specificity.

"Nonspecific" retention on glass-bead columns of about 50% of the spleen cells has been reported by Wigzell and Andersson.[12] In the work reported here, we found that 94% of the spleen cells were recovered after a second passage through clean glass beads and thus that the "nonspecific" separation on glass beads is indeed a cell fractionation, probably due to factors related to the net electrical charge on the cell surface.

The transfer of 8×10^6 filtered cells generated antibody titers about 4-fold lower than the transfer of an equal number of unfiltered cells, after the first immunization with the acidic DNP-901. This observation suggests retention of cells preferentially selecting the acidic immunogen. On the other hand, after secondary stimulation, no significant difference was detected in the titers of antisera from mice inoculated with filtered or unfiltered cells. At least two possible explanations could be given for this: (1) a small number of the more basic cells passed through the column, and subsequently proliferated after boosting; or (2) a greater proportion of the more acidic cells than was expected responded to the negatively charged immunogen, and elicited antibodies of a more acidic nature. Indeed, antibodies to the acidic DNP-901 produced in recipients of filtered cells were mainly in the second, more acidic fraction eluted from DEAE-Sephadex. This finding is in contrast to results obtained by chromatographing antisera from recipients of unfiltered cells, since in the latter case, similarly to antisera of unirradiated immunized mice, the majority of antibodies was detected under the first, more basic peak, as expected (Table 1).

The experiments in which lower numbers of filtered or unfiltered spleen cells were inoculated confirm the cellular basis for the net charge phenomenon, since a reduction in the percentage of positive sera was observed only in recipients of filtered cells immunized with the acidic immunogen (Table 2). Since it was previously demonstrated[1] that part of the antibodies ($\sim$10%) to the acidic DNP-901 were found in the second fraction eluted from DEAE-Sephadex, the percentage of responses to this immunogen would have been expected to be reduced to zero provided there were enough cells of the more basic type to generate a response.

It is noteworthy that the population of cells reactive with the basic DNP-912 did not appear to be significantly enriched after glass-bead column filtration. This could have been due to another limiting cell type with a function not related to net charge.

The data presented in this report provide the first direct experimental evidence for the cellular basis of net charge correlation between immunogens and the antibodies produced.

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Abbreviations: Dnp, dinitrophenyl; DNP-901, the acidic dinitrophenylated copolymer Dnp-poly $Glu^{83}Lys^{12}Tyr^{5}$; DNP-912, the basic dinitrophenylated copolymer Dnp-poly Glu^{19}-$Lys^{75}Tyr^{6}$.

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