Immunology 1986 **58** 541–544

Affinity maturation in the arsonate system: lack of dominance of high-affinity antibody subpopulations

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Accepted for publication 20 March 1986

SUMMARY

Affinity maturation was studied by the analysis of the kinetics of the appearance of antibody subpopulations with different affinities during the immune response, using an hapten-inhibition ELISA. The immune response in KLH-Ar-immunized A/J mice was used as a model system. Five antibody subpopulations of different affinity (10^3–10^7 M^{-1}) could be detected, the relative concentrations of which changed during affinity maturation. The high-affinity antibody subpopulations did not represent the major fraction at any stage during affinity maturation. The appearance of the highest affinity subpopulation (10^7 M^{-1}), despite exhibiting relative concentrations no higher than 12%, produced an important increase in average affinity. On the other hand, its disappearance at the end of the maturation process could explain the average affinity decrease observed at this stage. Our results indicate that affinity maturation cannot be explained by the dominance of high-affinity clones, as proposed by Siskind & Benacerraf (1969). The increase in affinity could rather be due to the progressive appearance of low percentages of high-affinity clones, which are not present in the primary response and never become dominant.

INTRODUCTION

The existence of a progressive increase in the average association constant (affinity) for the haptenic determinant during immune response development was initially described by Eisen & Siskind (1964). This phenomenon is known as affinity maturation of the immune response, and has been confirmed by several authors (Werblin *et al.*, 1973; Goidl, Barondess & Siskind, 1975; Conger, Lewis & Goodman, 1981; Rothstein & Gefter, 1983). Due to the importance of affinity maturation in the immune response, several hypotheses have been proposed to explain the molecular basis of this maturation process. One, postulated by Siskind & Benacerraf (1969), suggests that selective stimulation of high-affinity antibody-producing clones may result from the progressive decrease in antigen concentration after immunization. To verify this hypothesis, knowledge of antibody affinity distribution kinetics was needed (Eisen & Siskind, 1964). However, the experimental approach to this problem has proven difficult to conduct. A computer-based iterative curve-fitting approximation technique to process equilibrium dialysis data was developed by Werblin & Siskind (1972). Using this method, Werblin *et al.* (1973) observed at the beginning of the response a heterogeneous symmetric distribution of low-affinity antibodies followed by a gradual average affinity increase with a more homogeneous distribution pattern, where high-affinity subpopulations became the major fraction. At the end of the maturation process, an average affinity decrease was described, together with a more heterogeneous distribution, where high-affinity subpopulations were no longer predominant. Low-affinity subpopulations were detected throughout the maturation process, although their concentration decreased when average affinity increased. These observations were considered to confirm the hypothesis regarding the increase of antibody affinity with time (Siskind & Benacerraf, 1969). However, the theory did not account for the affinity decrease observed late in the response (Goidl *et al.*, 1975; Pini *et al.*, 1980; Tasiaux *et al.*, 1976; Werblin *et al.*, 1973). More recently, another hypothesis has been proposed suggesting that the decrease results from the production of anti-idiotype antibody against high-affinity predominant antibodies (Tasiaux *et al.*, 1976).

In the present report, affinity maturation is investigated using an ELISA developed in our laboratory (Nieto *et al.*, 1984) that allows the direct experimental determination of antibody affinity distributions. Using this approach, we have observed an average affinity increase during the first phase of the immune response due to the appearance of high-affinity subpopulations. Unexpectedly, these high-affinity antibody subpopulations never became predominant. On the contrary, they decreased late in the response, which probably accounts for the decrease in average affinity observed late in the immune response.

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MATERIALS AND METHODS

Animals

A/J mice were originally obtained from OLAC Ltd (Bicester, Oxon, U.K.) and have been bred in our laboratory. Mice were 6 weeks old at the time of primary immunization.

Antigens and immunization

KLH (Calbiochem Behring Corp., La Jolla, CA) and BSA (Merck, Darmstadt, FRG) were conjugated with arsanylic acid (Fluka 15 AG, Buchs, Switzerland) as described by Nisonoff (1967). KLH-Ar emulsified (1 : 1) in complete Freund's adjuvant (Gibco, Grand Island, NY) was used as immunogen. Mice were immunized by injecting 0·2 ml of emulsion intraperitoneally with 500 μg of KLH-Ar on Day 0 and with 50 μg KLH-Ar on Day 24. Animals were bled via the retro-orbital venous plexus on Days 24, 44, 62, 76, 96 and 167.

Determination of anti-Ar antibody concentrations

The ELISA used for determination of anti-Ar antibodies concentration has been described elsewhere (Nieto *et al.*, 1984). Briefly, polystyrene plates (Dynatech M-129-5, Kloten, Switzerland) were coated with 100 μl/well of a 10 μg/ml BSA-Ar36 solution in 0·1 M carbonate–bicarbonate buffer, pH 9·6 (moist chamber, room temperature, overnight). After discarding the coating solution, 200 μl/well 0·05% Tween 20 and 1% BSA in PBS (PBS-T-S) were added and the plates incubated for 1 hr at room temperature (moist chamber). After washing, immune sera diluted in PBS-T-S (100 μl/well) were added. After 3 hr at room temperature (moist chamber), the plates were washed and the appropriate dilution of rabbit anti-mouse IgG(H+L) conjugated to horseradish peroxidase (Nordic, Tilburg, The Netherlands) in PBS-T-S was added. The plates were incubated overnight at 4° in a moist chamber. After washing, enzymatic activity was determined according to Ngo & Lenhoff (1980). Affinity-purified antibody used as anti-Ar standard in the assay was kindly provided by Dr A. Nisonoff, Brandeis University, Waltham, MA. ELISA data are expressed as equivalent concentrations of this standard.

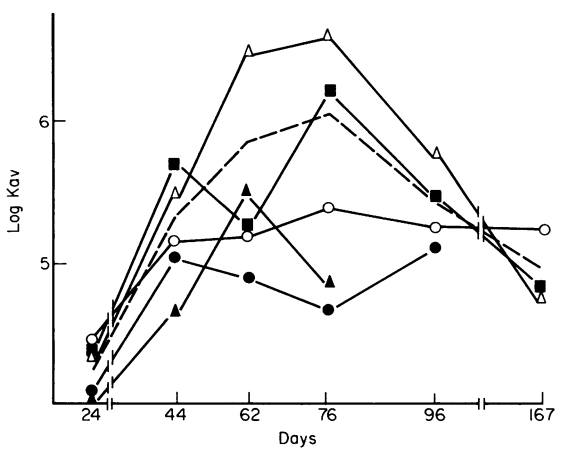

Figure 1. Affinity maturation: average affinity(Kav) kinetics is shown as log Kav changes observed at different times (days) after immunization. Results are plotted for individual mice [continuous line: no. 1 (●); no. 2 (○); no. 3 (▲); no. 4 (■); no. 5 (△)], and the mean values calculated from them (broken line) are plotted separately.

Determination of affinity distribution

In order to measure the apparent affinity constant (aK) of each subpopulation and the average affinity constant (Kav) of an immune serum, a similar protocol to the anti-Ar antibody determination was used. This technique has been extensively discussed in a previous paper (Nieto *et al.*, 1984). Plates were coated with several BSA-Ar36 concentrations (0·03, 0·1, 0·5, 1 and 10 μg/ml). Serum samples were mixed with Tyr-Ar solutions of different concentrations (10^{-2}–10^{-8} M) and added to the coated plates. Subpopulation percentages were calculated from the bound anti-Ar concentration increments observed between two successive solid-phase BSA-Ar36 concentrations. Apparent affinity constant of each subpopulation were calculated as 1/(hapten concentration producing 50% inhibition at the corresponding solid-phase BSA-Ar36 concentration). The average affinity constant (Kav) was calculated as the weight average of aK values corresponding to all subpopulations in a given serum.

RESULTS

Affinity maturation

Five mice were individually analysed for Kav and affinity distribution changes during the immunization process against KLH-Ar. They were immunized intraperitoneally with 500 μg KLH-Ar in CFA and boosted 24 days later with 50 μg KLH-Ar in CFA also intraperitoneally. They were bled on Day 24 (primary response) and on Days 44, 62, 76, 96 and 167 (secondary response). Both individual and mean average affinity changes are shown in Fig. 1. An affinity increase, subject to broad individual variation, was observed. The greatest change in the Kav value was observed at the beginning of the secondary response. A decrease in affinity during the later stages of the response was seen, as described in other experimental models (Werblin *et al.*, 1973; Goidl *et al.*, 1975; Tasiaux *et al.*, 1976; Pini *et al.*, 1980).

Although the highest antibody concentration was observed on Day 44 (data not shown), average affinity progressively increased until Day 76, and no correlation could be observed between anti-Ar antibody concentration and Kav.

Subpopulation analysis during affinity maturation

The distribution of affinities of antibodies in individual mice, immunized as described above, was determined by hapten-inhibition ELISA (Nieto *et al.*, 1984) and is shown in Fig. 2 (Columns 1–5, individual mice; Column 6, grouped mice). The subpopulations detected were in the 10^3–10^7 M^{-1} range. This is consistent with the serum average affinity constant values described in this experimental system (10^4–10^6 M^{-1}) and measured by equilibrium dyalisis (Kapsalis, Tung & Nisonoff, 1976; Kresina, Rosen & Nisonoff, 1982), fluorescence quenching (Rothstein & Gefter, 1983) and hapten inhibition PFC (Conger *et al.*, 1981).

As has already been described (Nieto *et al.*, 1984), different antibody subpopulations can be defined according to significant differences in aK. Thus, subpopulations detected in all bleedings of all mice (Fig. 2) were grouped in sets in which the aK differed by one log unit. Their percentages are plotted in Fig. 3. Twenty-four days after primary immunization only low-affinity subpo-

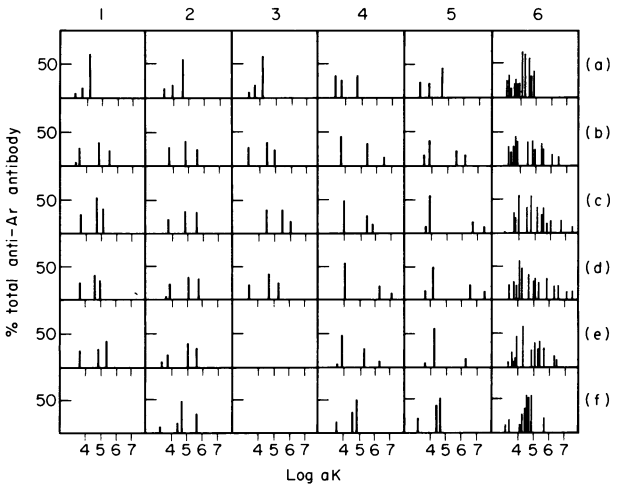

Figure 2. Affinity distribution during maturation: percentages (ordinates) of subpopulations of different affinity observed in individual (Columns 1–5) and grouped (Column 6) mice are plotted against the logarithm of their apparent affinity constant (abcissae) for each bleeding. Bleedings were done on Days 24(a); 44(b); 62(c); 76(d); 96(e) and 167(f), with antigen administration on Days 0 and 24.

pulations (10^3 and 10^4 M^{-1}) could be detected, whilst high-affinity subpopulations (10^5, 10^6 and 10^7 M^{-1} appeared after booster immunization, producing an increase in both average affinity (Fig. 1) and heterogeneity (Fig. 2). The presence of all subpopulations at varying relative concentrations was observed in all secondary response sera. A partial decrease in the percentages of the low-affinity subpopulations together with an increase of those of high affinity was observed in the secondary response, when compared with the primary response (Fig. 3). There was a correlation between a decrease in a certain subpopulation and the increase in one with an immediately higher affinity (Fig. 3) (i.e. decrease of the 10^4 M^{-1} subpopulation in the secondary response correlates with an increase of the 10^5 M^{-1} subpopulation; a decrease in the 10^3 M^{-1} subpopulation, observed on Day 62, corresponds to an increase in the 10^4 M^{-1} subpopulation).

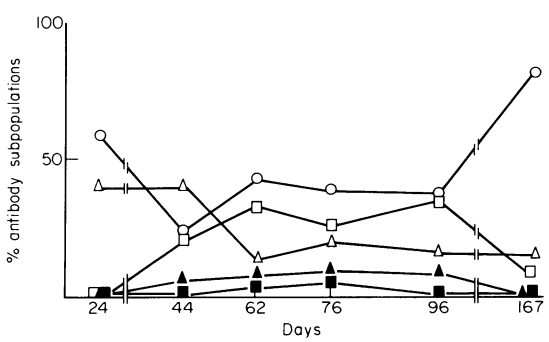

Figure 3. Evolution pattern of subpopulation percentages during maturation: the percentages of the different affinity subpopulations (ordinates) are plotted separately against the different times after immunization (abcissae). Each point corresponds to the average value from all mice [10^3 M^{-1} (△); 10^4 M^{-1} subpopulation (○); 10^5 M^{-1} subpopulation (□); 10^6 M^{-1} subpopulation (▲); 10^7 M^{-1} subpopulation (■)].

High-affinity subpopulations (10^5, 10^6, and 10^7 M^{-1}) appeared during the secondary response (Fig. 2), and although their percentages increased progressively, these subpopulations never became dominant. Despite this lack of dominance, the appearance of the high-affinity subpopulation seems to be responsible for the Kav increase (Fig. 1). It is worth mentioning that the 10^7 M^{-1} subpopulation was present only in mice no. 4 (12%) and no. 5 (11%), which had the highest affinity values (Fig. 1).

Late in the response a decrease in Kav was observed. This decrease is due to the disappearance of high-affinity subpopulations (10^7 M^{-1} on Day 96 and 10^6 M^{-1} on Day 167). These sequential studies show the progressive increase in the high-affinity subpopulations, which in spite of being low in percentage, have a marked influence on the Kav increase during the secondary response.

DISCUSSION

Affinity maturation has been analysed by the measurement of antibody subpopulations of different affinity during immunization (primary and secondary response). In spite of the individual variability observed in affinity data (Fig. 1), which has also been observed by other authors (Jarvis *et al.*, 1982), a similar subpopulation evolution pattern can be observed during maturation in all mice (Fig. 2). We have experimentally verified that average affinity variations depend on changes in the relative concentration of different subpopulations and sometimes on the appearance or absence of some of them (Figs 2 and 3) (Werblin *et al.*, 1973). Thus, the presence of a 10^7 M^{-1} subpopulation establishes a large average affinity difference between mice no. 4 and no. 5 and the rest on Day 76 (Fig. 1). This difference shows that high-affinity subpopulations exert a marked influence on average affinity, although their relative concentrations may not be high. Moreover, the changes in the affinity subpopulations seen in Fig. 3 show that the increase in Kav from Day 62 to Day 76 (Fig. 1) does not result from a further increase in the 10^5 M^{-1} subpopulation percentage, but rather from the appearance of low levels of the 10^6 and 10^7 M^{-1} subpopulations (Fig. 3).

From these results, it can be concluded that the continuous expansion of high-affinity clones is not necessary for the Kav increase, which is due mainly to the appearance of low percentages of very high affinity subpopulations (10^6 and 10^7 M^{-1}). Although only a single immunization protocol has been used here, a similar conclusion can be deduced from careful analysis of anti-fluorescyl rabbit antibody dissociation rates presented by Herron & Voss (1983), who used a very different model (fluorescein, rabbit, . . .). If we evaluate the degree of heterogeneity according to the number of different species of molecules of different affinity (Siskind & Benacerraf, 1969), we can observe during the maturation process (Fig. 2) a heterogeneity increase parallel to the average affinity increase. This heterogeneity increase corresponds to an increase in the number of different subpopulations present during the response. At the end of the response there was a decrease in the heterogeneity due to the disappearance of the highest affinity subpopulations. Together with the decrease in the heterogeneity, a decrease in the Kav during the final stage of the response was also present. This observed decrease in Kav has been widely described (Goidl *et al.*, 1975; Pini *et al.*, 1980; Tasiaux *et al.*, 1976; Werblin *et al.*, 1973), but so far it has not been explained satisfactorily. The

hypothesis of cell selection by the antigen (Siskind & Benacerraf, 1969) can explain the increase of Kav but is unable to explain its decrease at the end of the response. On the basis of the predominance of the high-affinity subpopulation predicted by this hypothesis, it has been proposed (Tasiaux *et al.*, 1976) that anti-idiotype formation against the predominant subpopulation may be responsible for the Kav decrease. Our data do not support this hypothesis. The highest affinity subpopulation (10^7 M^{-1}) is present at a maximum percentage of 12%, whereas the 10^4 and the 10^5 M^{-1} subpopulations have percentages in the 30–40% range. If a decrease in the level of these predominant subpopulations does not appear to be due to anti-idiotype regulation, such a phenomenon would not be expected in the case of higher affinity subpopulations, the levels of which are much lower. However, it can not be ruled out that microheterogeneity inside the 10^4–10^5 M^{-1} subpopulations is higher than in the 10^7 M^{-1} subpopulation. Thus, the levels of different clones inside the 10^4–10^5 M^{-1} subpopulations would be insufficient to stimulate anti-idiotype formation. Nonetheless, up to now there are no data available to support this hypothesis. On the other hand, Berek, Griffiths & Milstein (1985) have recently presented data suggesting that high-affinity subpopulations are generated by somatic mutation of originally recruited germ line genes and by recruitment of other germ line gene combinations. Obviously, both somatic mutation and new germ line gene combinations producing high-affinity antibodies probably occur infrequently. Consequently, when antigen concentration decreases, the antigen is not equally available and only the widely distributed clones are stimulated. In a situation like this, high-affinity clones would probably not be stimulated due to a lack of antigen. This results in a decrease in their concentrations, giving rise to a decay in the average affinity.

In conclusion, we have verified that the increase in the average affinity during the maturation process is not due to the dominance of the highest affinity clones. Having taken into account this lack of dominance, the average affinity decrease observed late in the response does not seem to be due to anti-high-affinity idiotype synthesis. On the other hand, it is more simple to suppose that these high-affinity clones are unequally distributed, and that when the antigen concentration and availability fall, these clones are no longer stimulated.

ACKNOWLEDGMENT

This work was supported by Grant No. 83/0817 from Fondo de Investigaciones Sanitarias de la Seguridad Social.

REFERENCES

Berek C., Griffiths G.M. & Milstein C. (1985) Molecular events during maturation of the immune response to oxazolone. *Nature (Lond.)*, **316,** 412.

Conger J.D., Lewis G.K. & Goodman J.W. (1981) Idiotype profile of an immune response. I. Contrast in idiotypic dominance between primary and secondary responses and between IgM and IgG plaque forming cells. *J. exp. Med.* **153,** 1173.

Eisen M.N. & Siskind G.W. (1964) Variation in affinities of antibodies during the immune response. *Biochemistry*, **3,** 996.

Goidl E.A., Barondess J.J. & Siskind G.W. (1975) Studies on the control of antibody synthesis. VII. Change in affinity of direct and indirect plaque forming cells with time after immunization in the mouse: loss of high affinity plaques late after immunization. *Immunology*, **29,** 629.

Herron J. & Voss E.W. (1983) Analysis of heterogeneous dissociation kinetics in policlonal populations of rabbit-antifluoresceyl-IgG antibodies. *Molec. Immunol.* **20,** 1323.

Jarvis M.R., Casperson G.F., Kranz D.M. & Voss E.W. (1982) Affinity maturation of NZB and Balb/cV mice anti-fluoresceil response. *Molec. Immunol.* **19,** 525.

Kapsalis A.A., Tung A.S. and Nisonoff A. (1976) Relative combining affinities of anti-*p*-azophenylarsonate antibodies bearing a cross reactive idiotype. *Immunochemistry*, **13,** 783.

Kresina T.F., Rosen S.M. & Nisonoff A. (1982) Degree of heterogeneity of binding specificities of antibodies to the phenylarsonate group that share a common idiotype. *Molec. Immunol.* **19,** 1433

Ngo T.T. & Lenhoff H.M. (1980) A sensitive and versatile chromogenic assay for peroxidase and peroxidase coupled reactions. *Analyt. Biochem.* **105,** 389.

Nieto A., Gayà A., Jansà M., Moreno C. & Vives J. (1984) Direct measurement of antibody affinity distribution by hapten-inhibition enzyme immunoassay. *Molec. Immunol.* **21,** 537.

Nisonoff A. (1967) Coupling of compounds to proteins. In: *Methods in Immunology and Immunochemistry* (eds C.A. Williams and M. W. Chase), Vol. I, p. 120. Academic Press, New York.

Pini C., Di Felice G., Neri R., Mancini C., Vigari G. & Doria G. (1980) Oscilations of IgM antibody affinity at the level of single immunocytes. *J. Immunol.* **125,** 1349.

Rothstein T.L. & Gefter M.L. (1983) Affinity analysis of idiotype-positive and idiotype-negative Ars-binding hibridoma proteins and Ars immune sera. *Molec. Immunol.* **20,** 161.

Siskind G.W. & Benacerraf B. (1969) Cell selection by antigen in the immune response. *Adv. Immunol.* **10,** 1.

Tasiaux N., Leuwenkroon R., Bruyns C. & Urbain J. (1976) Possible occurrence and meaning of lymphocytes bearing auto anti-idiotypic receptors during the immune response. *Eur. J. Immunol.* **8,** 464.

Werblin T.P., Kim Y.T., Quagliata F. & Siskind G.W. (1973) Studies on the control of antibody synthesis. III. Changes in the heterogeneity of antibody affinities during the course of the immune response. *Immunology*, **24,** 477.

Werblin T.P. & Siskind G.W. (1972) Distribution of antibody affinities: technique of measurement. *Immunochemistry*, **9,** 987.