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Increased frequency of rheumatoid factor precursor B lymphocytes after immunization of normal adults with tetanus toxoid

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SUMMARY

Vaccination of normal adults with tetanus toxoid induced a two–three-fold rise in the frequency of IgM anti-IgG (rheumatoid factor, RF) B lymphocytes inducible by the polyclonal B cell activator, Epstein–Barr virus. The increase in IgM-RF precursors occurred earlier, was greater in magnitude, and was more sustained than the change in plasma IgM-RF. It was associated with a rise in total IgM levels, and correlated positively with the magnitude of the IgG anti-tetanus antibody response, but not with levels of circulating immune complexes. The ability of apparently innocuous infections and immunizations to increase the frequency of IgM-RF precursor B lymphocytes may be the reason for the previously noted expansion in this autoreactive B cell pool between birth and adulthood.

INTRODUCTION

The incidence of humoral autoimmune diseases generally rises with age, and correlates with an increase in the frequency and mean titres of serum autoantibodies (Mackay, Whittingham & Mathews, 1977). Recently, Fong *et al.* (1981) reported that the frequency of peripheral blood B lymphocytes inducible by Epstein–Barr virus (EBV) to the production of IgM rheumatoid factor (IgM-RF precursor B lymphocytes) expanded several fold between birth and adulthood. Because T cell influences are felt to be minimal when EBV stimulation is carried out under limiting dilution conditions, the results indicated an age associated rise in numbers of IgM-RF precursor B cells, not a change in T cell suppression.

The factors potentially causing an expansion in IgM-RF precursor B cells between birth and adulthood in normal people have not been defined. In certain strains of mice an age-dependent increase in IgM-RF autoantibodies was shown by Van Snick & Masson (1979, 1980) to require exposure to unknown environmental agents. A few studies in man have reported rises in *in vivo* autoantibody titres following vaccination of apparently normal individuals (Svec & Dingle, 1965; Rhodes *et al.*, 1969; Kreisler, Hirata & Terasaki, 1970). Vaccination is a potent immunological stimulus experienced by most individuals in developed countries and may function as a potential inducing agent of autoantibody production.

In the present investigation, the frequency of IgM-RF precursor B lymphocytes inducible by

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EBV was measured before and after tetanus toxoid vaccination of normal adults. As early as 3 days after vaccination, the frequency of IgM-RF precursor cells rose two–three-fold. The increase persisted at 7 and 18 days after immunization. Changes in plasma IgM-RF were less marked, and were more transient. The increase in IgM-RF precursor B lymphocytes following vaccination was not accompanied by the appearance of circulating immune complexes, but it did positively correlate with the IgG tetanus antibody response.

MATERIALS AND METHODS

Vaccination of human subjects. Eleven healthy adult volunteer subjects were entered in the study. Data from three of the subjects was excluded because their EBV cultures did not meet limiting dilution conditions (see below). The mean age of the eight remaining subjects (five females, three males) was 27 years (range = 21–34 years). The duration since their last tetanus vaccination was 5–10 years in five subjects, and greater than 10 years in three subjects. Each person received 0·5 ml tetanus toxoid (aluminium phosphate adsorbed, Lederle Laboratories, Pearl River, New York, USA) intramuscularly.

Lymphocyte isolation and EBV infection. Samples of heparinized blood were obtained from the subjects just before vaccination (0 day), and 3 days, 7 days, and 18 days thereafter. Mononuclear cells were isolated by Ficoll-Hypaque density sedimentation and frozen in liquid N_2 using standard techniques (Böyum, 1968; Birkeland, 1980). Cells were kept frozen for 30–50 days before EBV infection. Plasma was also frozen and stored for later analysis. Mononuclear cells were infected with EBV as previously described (Fong *et al.*, 1981). With each sample, we initiated 60 EBV infected and 20 control cultures containing 10^5 cells each, in a volume of 0·2 ml RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Supernatants were collected after a 28 day incubation.

IgM-RF assay. Supernatants and plasmas were analyzed for IgM-RF and total IgM using standard ELISA modifications of our previously described radioimmunoassays (Fong *et al.*, 1981). For the final antibody coating, alkaline phosphatase labelled goat anti-human IgM (1 μg/ml) (Kirkegaard and Perry, Gaithersburg, Maryland, USA) was substituted for the radiolabelled anti-IgM previously used. After a 5 hr incubation at 37°C and four washings, alkaline phosphatase substrate (*p*-nitrophenyl phosphate, disodium, Sigma, St Louis, Missouri, USA) in 0·05 M carbonate/bicarbonate buffer, pH 9·8, was added at a concentration of 1 mg/ml to all the wells, and then reacted for 60 min. The optical density of the wells was then read at 405 nm. A standard curve for IgM-RF and for total IgM utilized a monoclonal IgM protein with anti-IgG antibody activity, as described earlier (Tsoukas *et al.*, 1980). A microtitre well was considered positive for IgM-RF if the OD_{405} was at least three standard deviations above the background OD_{405} of wells reacted with just RPMI with 10% FCS.

The fraction of microcultures positive for EBV-induced IgM-RF ranged from 0·2 to 0·6 in eight subjects. Using this fraction, the relative frequencies of IgM-RF producing cells in the various pre- and post-vaccination samples were determined utilizing the Poisson distribution $P_{(0)} = e^{-\phi x}$, where $P_{(0)}$ is the proportion of non-responding wells, x is the number of cells cultured per well and ϕ is the relative responding cell frequency (Lefkovits, 1972). Three subjects had to be excluded because their fraction of positive IgM-RF microcultures was too high ($> 0{\cdot}9$) and therefore did not meet limiting dilution conditions.

Measurement of IgG anti-tetanus toxoid antibody, and plasma IgM levels. Plasma IgG anti-tetanus toxoid antibody was measured by an ELISA method developed in this laboratory. Wells of a 96 well vinyl assay plate were coated with 100 μl of tetanus toxoid diluted 1:80 in borate-buffered saline, pH 8·2 (BBS) by incubating at 37°C for 18 hr. Unbound material was aspirated and the wells washed with BBS three times. One hundred microlitres of serial dilutions of plasma (10^{-2}–10^{-4} dilution range) was then added and reacted for 18 hr at 4°C. After the wells were aspirated and washed three times, the final antibody coating was done as described above for RF except that alkaline phosphatase labelled goat anti-human IgG was used instead of labelled anti-human IgM. The standard curve was derived from serial dilutions of a standard human tetanus

immune globulin (Hypertet®, 250 animal protection units/ml, Cutter Laboratories, Berkeley, California, USA). The negative control was a plasma sample from a patient with agammaglobulinaemia. IgG anti-tetanus antibody activity in plasma was reported in the same units as the Hypertet® standard, i.e., animal protection units/ml. Plasma IgM levels were determined by a radial immunodiffusion assay using a Partigen plate (Behring Diagnostics, Somerville, New Jersey, USA).

Circulating immune complexes. Plasma samples were analyzed for circulating immune complexes by the ^{125}I-Clq solution phase binding method, and by the Raji cell assay (Zuber *et al.*, 1976; Theofilopoulos, Wilson & Dixon, 1976).

RESULTS

Vaccination induced changes in IgM-RF precursors, plasma IgM-RF, total IgM and IgG anti-tetanus antibody

Table 1 summarizes the mean IgM-RF precursor frequencies and the plasma levels of IgM-RF, total IgM and IgG anti-tetanus toxoid, before and after vaccination of the eight normal subjects. Prior to immunization, the relative frequency of IgM-RF precursor B lymphocytes was $2{\cdot}4 \pm 1{\cdot}0$ per 10^6 cells (s.e.m.), a value that falls within the range previously reported for normal adults. By 3 days after vaccination the IgM-RF precursor frequency had risen to 278% of the starting value, and remained elevated at days 7 and 18 post-vaccination, although the elevation at 18 days was not statistically significant.

With the sensitive ELISA assay, small amounts of IgM-RF were detected in the plasmas of all the normal subjects prior to vaccination (mean $3{\cdot}92 \pm 0{\cdot}66$ μg/ml). Three days after immunization with tetanus toxoid, these levels had not changed. However, at 7 days. IgM-RF levels had risen to $173 \pm 32\%$ of the baseline. By 18 days after vaccination, the amounts of plasma IgM-RF levels had returned to pre-immunization values.

The subjects, as a whole, demonstrated a good anti-tetanus toxoid antibody response to the vaccination. The amounts of IgG anti-tetanus toxoid antibody in the plasmas rose to $244 \pm 59\%$ of starting levels by day 7 after vaccination, and remained elevated at day 18. The mean concentration of IgM in the plasma concentrations increased to $139 \pm 21\%$ of the baseline level at day 3 following vaccination, thereafter returning to pre-vaccination value.

Figs 1a & 1b show representative examples of changes in peripheral blood IgM-RF precursor B cell frequency, plasma IgM-RF level, and total plasma IgM concentration in two individuals immunized with tetanus toxoid. In both cases, the increase in IgM-RF precursor B cells occurred

Table 1. Increase in IgM-RF precursor B cells, plasma IgM-RF, total IgM and IgG anti-tetanus toxoid after tetanus vaccination

Parameter measured	Percentage of starting value*			
	0 days	3 days†	7 days	18 days
IgM-RF precursor frequency	100	278 ± 81‡	289 ± 79‡	280 ± 119
Plasma IgM-RF	100	103 ± 17	173 ± 32	112 ± 20
Plasma IgM	100	139 ± 21‡	115 ± 16	94 ± 6
Plasma IgG anti-tetanus toxoid	100	n.d.	244 ± 59‡	368 ± 139‡

* $\frac{\text{Individual level at day indicated}}{\text{Individual level at day 0}} \times 100$; value given is mean % ± s.e.m. of eight subjects.

0 day absolute values: IgM-RF precursor frequency, $2{\cdot}40 \pm 1{\cdot}16$ per 10^6 cells; plasma IgM-RF, $3{\cdot}92 \pm 0{\cdot}66$ μg/ml; plasma IgM, $1{\cdot}53 \pm 0{\cdot}14$ mg/ml.

† Days post-vaccination.

‡ $P < 0{\cdot}05$ compared to 0 day value (Mann–Whitney Test).

n.d. = Not done.

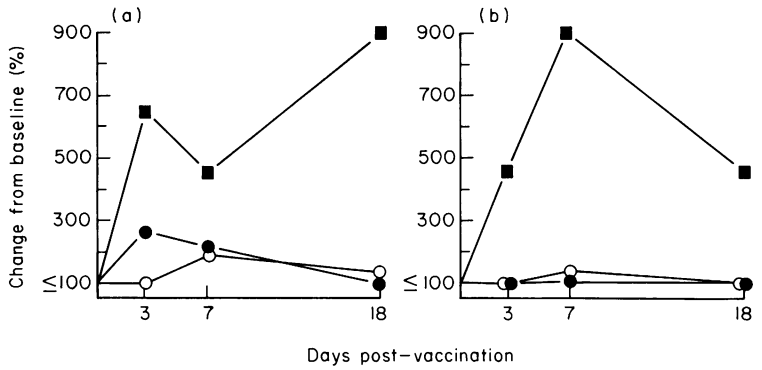

Fig. 1. Representative examples of changes in peripheral blood IgM-RF B cell frequency, plasma IgM-RF, and plasma IgM concentration after tetanus toxoid vaccination in two individuals, A and B. ■ = IgM-RF B cell frequency; ○ = IgM-RF; ● = total IgM.

earlier, was greater in magnitude, and was more sustained than the change in plasma IgM-RF levels.

Immunospecificity of IgM-RF

It was important to determine whether or not microcultures were positive for IgM-RF activity simply because of increased total IgM production in the same samples. Therefore, IgM levels in supernatants from 100 RF positive wells and 80 RF negative wells were compared. The mean level of IgM in the positive wells was no different from that of the negative wells ($12{\cdot}5 \pm 0{\cdot}9$ μg/ml vs $14{\cdot}0 \pm 2{\cdot}0$ μg/ml, respectively).

The specificity of IgM-RF in the culture supernatants was also tested by adsorbtion with human IgG. The supernatants of positive IgM-RF producing microcultures were pooled for each individual's 60 well microassay plate. Sufficient supernatant was available in 25 different plates to allow for analysis. Twenty of these pooled supernatants had IgM-RF activity that was adsorbed specifically by IgG coated plates, but not by plates coated with BSA. The mean drop in IgM-RF after a single adsorption was $42 \pm 5\%$. A single adsorption of a purified monoclonal IgM protein with anti-IgG antibody activity at similar concentrations yielded a $68 \pm 2\%$ decrease in IgG binding activity.

Correlation between changes in IgG anti-tetanus toxoid antibody and the frequency of IgM anti-IgG producing cells

The magnitude of the rise in IgG anti-tetanus toxoid antibody following vaccination correlated positively with the increase in IgM-RF precursor frequency ($r = 0{\cdot}65$, $P < 0{\cdot}01$). In contrast, there was no correlation found between either the change in plasma IgM-RF, or total IgM, and precursor frequency. Furthermore, there was a lack of correlation between any two of the three plasma parameters measured.

Determination of immune complexes

Vaccination was not associated with the observable appearance of circulating immune complexes as measured by either the Raji cell (normal < 12 μg AHG equivalents per ml) or the Clq assay (normal < 13%).

DISCUSSION

Tetanus toxoid vaccination of normal adults caused a two–three-fold increase in the relative frequency of IgM-RF precursor B cells in peripheral blood inducible by the T cell-independent,

polyclonal B cell activator, EBV. By 3 days after vaccination, the IgM-RF B cell frequency had risen significantly, and this increase persisted as late as 18 days post-vaccination. In contrast, plasma IgM-RF levels increased only at 7 days after vaccination, returning to baseline thereafter. At no time were circulating immune complexes detectable in plasma, as measured by either the Clq or Raji cell assay. However, the immunization did induce an early and transient increase in total plasma IgM levels.

Previous investigations have established that vaccination of normal people occasionally leads to the appearance of IgM-RF in plasma (Svec & Dingle, 1965; Rhodes *et al.*, 1969). However, the assay of plasma IgM-RF yields only limited information concerning the effects of vaccination on autoantibody production. A proportion of newly synthesized IgM-RF probably is cleared from the circulation after binding serum IgG, and is not detected by routine assays. Additionally, not all the B lymphocytes with surface IgM-RF actually contribute to the ongoing synthesis of the autoantibody. Thus, as reported here, many normal adults with little or no IgM-RF in plasma nevertheless have an appreciable frequency of EBV inducible RF precursor cells in the blood. These presumably derive from a memory B lymphocyte pool. The above considerations may explain why the frequency of IgM-RF B cells inducible by EBV increased by 180% following tetanus vaccination, although plasma IgM-RF levels rose by only 70%.

Two likely causes for the increase in IgM-RF precursors following an immunological stimulus such as a vaccination, are autoimmunization by antigen–antibody complexes, and polyclonal B cell activation. An earlier kinetic analysis of the development of IgM-RF in patients with subacute bacterial endocarditis showed that circulating immune complexes usually appeared in plasma 1–2 weeks prior to IgM-RF (Carson *et al.*, 1978). This time course was consistent with the autoantibody being an immune response to the antigen–antibody complexes that were abundant in all the subjects. However, in the present study, tetanus toxoid vaccination of normal adults did not lead to detectable immune complexes in plasma, as measured by either the Raji cell or Clq assay. One cannot exclude the possibility that small amounts of tissue fixed immune complexes induced IgM-RF in the patients studied. However, the very early increase in IgM-RF precursor frequency concomitant with an elevation in total plasma IgM levels is more compatible with a connection between the RF response and polyclonal B cell activation.

Experiments by Brenner & Munro (1981) have shown that tetanus toxoid is an *in vitro* polyclonal B cell activator in immune individuals. One may reasonably predict that a similar phenomenon would occur *in vivo*. Agents like tetanus toxoid or common infectious organisms like viruses may be working *in vivo* to expand a pool of low frequency autoreactive B cells at the same time it is approximately expanding antigen specific B cells. One potential function of the IgM-RF produced during *in vivo* polyclonal immune responses may be to amplify the activity of low affinity IgG anti-bacterial antibodies. The IgM-RF synthesized *in vitro* following B cell activation binds poorly to monomeric IgG in solution but efficiently to IgG bound on a surface (Tanimoto *et al.*, 1975). Under the latter condition, IgM-RF is a potent activator of the complement system (Sabharwal *et al.*, 1982). In addition, because IgM-RF effectively renders low affinity IgG antibodies multivalent, it enhances their stability of interaction with antigen. Conceivably, IgM-RF synthesis in conjunction with polyclonal B cell activation may serve as a first line defence against bacterial or viral infection until significant, antigen specific antibody synthesis ultimately ensues. Furthermore, as demonstrated here, this polyclonal B cell activation may permanently affect the immune system by enhancing the numbers of IgM-RF precursor memory B cells. The ability of apparently innocuous infections and immunizations to increase the frequency of IgM-RF precursor B lymphocytes may be the cause for the previously noted expansion in this autoreactive B cell pool between birth and adulthood.

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