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Specific antibody synthesis *in vitro*

IV. The correlation of *in vitro* and *in vivo* antibody response to influenza vaccine in rhesus monkeys

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SUMMARY

Fourteen female monkeys (*Macaca mulatta*) received a trivalent influenza vaccine and antibody response was determined by a change in plasma antibody content (ELISA) before and after vaccine. Lymphocyte cultures were also established from these monkeys and the level of antibody response did not correlate with mitogen-induced lymphocyte blastogenesis or natural killer cell function. *In vitro* anti-influenza antibody synthesis, however, was found to correlate well with the *in vivo* response. That is, monkeys who were non-responders, as determined by lack of change in plasma antibody content, were also non-responders *in vitro*. Accordingly, we believe that vaccine response is not necessarily a measure of immune competence but its measurement may, none the less, have clinical utility. The excellent correlation of *in vivo* and *in vitro* response provides predictive value for the *in vitro* test. Furthermore, because the correlation is good, the *in vitro* test may be useful as a tool in immunopharmacology and toxicology.

Keywords specific antibody influenza vaccine immune competence rhesus monkeys

INTRODUCTION

The antibody response is an integral component of normal immunity. It is currently understood that successful humoral immunity is dependent upon a complex network of cellular interactions (for review, see Teale & Klinman, 1984), and it is not unusual to observe deficient antibody responses in immunologically compromised hosts (Norden & Makinodan, 1973; Weitzman *et al.*, 1977; Mazaheri *et al.*, 1984). In old people, for example, diminished responses are observed after tetanus toxoid or influenza vaccine, when compared to the responses observed in young cohorts (Kishimoto *et al.*, 1980). We have been investigating this age-reduced response and developing strategies to enhance what appears to be deficient T-helper cell function (Price & Makinodan, 1972; Gillis *et al.*, 1981; Miller, 1986). We have utilized a microculture system to detect specific antibody synthesis *in vitro* and have used this system to test the capability of certain immunopharmacological agents to augment antibody production (Ershler *et al.*, 1984; Ershler, Moore & Socinski, 1984; Moore, Ershler & Hacker, 1984). Recently, we have expanded our studies to non-human primates, particularly rhesus monkeys (*macaca mulatta*), and currently report the responses of female monkeys of various ages both *in vitro* and *in vivo* after influenza haemagglutinin challenge (vaccine). Antibody responses were also correlated with other measures of immune competence.

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MATERIALS AND METHODS

Animals

Fourteen female rhesus monkeys (*Macaca mulatta*) of various ages, housed at the Wisconsin Regional Primate Research Center (WRPRC) or at the Harlow Laboratory (HL), were utilized for this study. Animals were selected on the basis of availability and the absence of known illness, medication or confounding experimental variables. Monkeys were housed singly or in groups in stainless steel cages with space, feeding and care in accordance with established guidelines for laboratory animal care. Health maintenance and evaluation are the responsibility of a well-trained full-time veterinary staff. All of the monkeys in our colony are tested twice annually for the presence of immunosuppressive retrovirus infection. Although 11% of the colony has antibody to a type D retrovirus, we have not observed any cases of immune deficiency (or Simian AIDS) developing in those animals. No animal in this study was positive for retrovirus by antibody determination.

Vaccine

Each monkey received a trivalent influenza vaccine composed of 3 μg each of three different influenza haemagglutinins. The vaccine used was a commercially available 1986/1987 subunit vaccine (Fluogen, Parke Davis, lot 02076P) comprised of purified haemagglutinin from the three prevalent viral strains. Earlier investigation in our laboratory indicated that this was an effective immunizing dose (data not shown). Peripheral blood was sampled prior to and periodically after vaccine for antibody response and lymphocyte functions.

Blood sampling and cell preparations

Approximately 7 cc of peripheral blood was obtained by venipuncture utilizing heparinized sterile vacutainer tubes. For this, the monkeys were restrained by one attendant and the blood was drawn from the femoral vein by a second. The sample was promptly taken to the laboratory for processing. Mononuclear cells (MNC) were prepared by density gradient centrifugation utilizing Ficoll–Hypaque. Plasma was removed and frozen (−70°C) for later antibody determinations. MNC present at the Plasma–Ficoll interface were aspirated, washed in sterile phosphate-buffered saline (PBS) and resuspended at a final density of 2×10^6 cells/ml in complete media which consists of RPMI 1640 (Gibco) with HEPES 25 mM (Gibco) and gentamicin 50 μg/ml (Gibco).

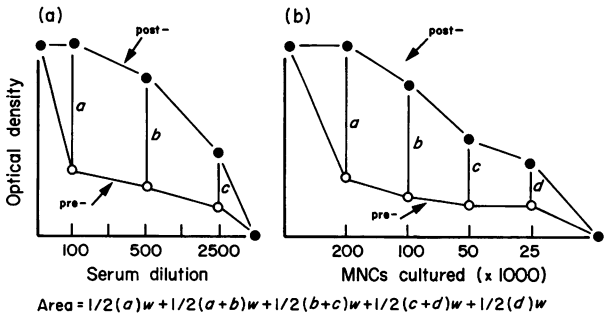

Fig. 1. Determination of antibody response. (a) *In vivo*. Plasma samples before and 4 weeks after influenza vaccine were analysed for anti-influenza antibody by ELISA and the adsorbance (optical density) at each of the indicated dilutions are plotted. The response was calculated by computing the area between the curves utilizing the indicated formula for area of a trapezoid as derived by Murphy *et al.* (1980). In the formula a, b and c are the difference in optical density at a 1:100, 1:500 and 1:2500 plasma dilution respectively, and *w* is arbitrarily assigned the value of 100. (b) Similar computation was undertaken for the *in vitro* antibody determination (MASELA) Lymphocyte cultures were established at four different concentrations and the antibody produced was also measured spectrophotometrically. The differences in optical density between those cultures established 1 week after vaccine and those established prior to vaccine were estimated by the same formula.

ELISA for antibody to influenza haemagglutinin

Plasma samples were thawed and tested for antibody content by an enzyme-linked immunosorbent assay (ELISA) as previously described (Ershler *et al.*, 1984; Ershler, Moore & Socinski, 1984). Briefly, for this analysis, influenza haemagglutinin (Connaught) diluted to a concentration of 0·15 μg/ml in bicarbonate buffer, pH 9·6, and 300 μl is added to each of the wells of micro-ELISA plates (Immulon-2, Dynatech, Alexandria, VA). After an overnight incubation at 4°C, the wells are washed with PBS–Tween (PBS–T) and serum samples (diluted (1:250, 1:1000 and 1:5000 in PBS–T) are added (200 μl). After a second overnight incubation at 4°C, the plates are again washed with PBS-T and alkaline phosphatase conjugated anti-IgG (human) (diluted 1:3000 in PBS-T) (Tago, Burlingame, CA) is added. After a final overnight incubation (4°C) and wash (PBS–T), the alkaline phosphatase remaining is quantified by measuring the cleavage product spectrophotometrically (Dynatech automated ELISA plate reader model 650) 30 min after substrate (*P*-nitrophenol phosphate, Sigma) is added. Multiple dilutions of a standard sample are prepared on each plate and optical densities for each sample are normalized arithmetically to the standard curve established for that plate. Also, all samples from an individual monkey were tested on a single plate to reduce the possibility of between-plate artifact. The alkaline phosphatase conjugated anti-IgG was an anti-human reagent (TAGO, Burlingame, CA), but preliminary investigations revealed cross-reactivity of this antibody with rhesus monkey IgG (data not shown). The antibody response was determined by calculating the area between the pre- and post-vaccine dilution curves as originally described by Murphy *et al.* (1980), and as depicted in Fig. 1.

In vitro anti-influenza antibody synthesis

Antibody production *in vitro* was assessed by the microculture antibody synthesis enzyme-linked assay (MASELA), as previously described (Ershler *et al.* 1984; Moore, Ershler & Hacker, 1984). MNC at different dilutions in media with 10% FCS were cultured in microtitre plates (Immulon 2, Dynatech) that had previously been coated (utilizing sterile technique) with influenza haemagglutinin (Connaught Laboratories) diluted to a concentration of 0·15 μg/ml in sterile buffered saline, pH 7·2. After six days of culture at 37°C in humidified 5% CO_2, cells were washed from the plates and the amount of antibody synthesized and secreted into the supernatant and adherent to bound influenza haemagglutinin was detected by ELISA as described above. Similarly, the response was reported as the area between the cell dilution curves from the samples established before and after vaccination (Fig. 1B). Appropriate controls, such as wells with media alone (±FCS), and wells with standard anti-influenza antibody were established on each plate. In humans the optimal interval between vaccination and establishing lymphocyte culture for detection of specific antibody to that vaccine was shown to be 6–7 days (Moore, Ershler & Hacker, 1984). This correlates with the presence of antigen-specific B cells in the peripheral blood (Stevens & Saxon, 1978, 1979), however we were not certain that this would be the case for monkeys. Therefore, for three monkeys, cultures were established at 2, 4, 6, 8 and 10 days after vaccine. Like humans, the optimal interval between vaccine and culture establishment was 6 days (data not shown).

Other lymphocyte functions

Mitogen response. MNC (10^5) were cultured in a volume of 200 μl of medium containing 25% heat-inactivated pooled human serum in microculture plates (Corning) for 3 days in the presence of mitogen. For these studies we utilized phytohaemagglutinin (PHA) (Sigma) at 40 μg/ml, concanavalin A (Con A) (Sigma) at 40 μg/ml and pokeweed mitogen (PWM) (Sigma) at 0·5 μg/ml. After 72 h of culture in humidified air with supplemented CO_2 (5%) at 37°C, 50 μl of media containing 1 μCi of ^{3}H-thymidine was added to culture. Eighteen hours later

Table 1. Anti-influenza vaccine response

Monkey	Age (yrs)	Sex	O.D. (pre)*	*In vivo* Ab Response (Area)†	*In vitro* Ab Response (Area)‡
M80169	7	F	0·659	0	27
M80115	7	F	0·555	125	42
M80035	7	F	0·379	210	62
M79151	8	F	0·902	81	23
AE17	10	F	0·616	415	111
AE79	10	F	0·193	179	48
PP30	16	F	0·602	456	ND
PP45	17	F	0·699	0	ND
PP52	17	F	0·902	81	ND
M1116	24	F	0·986	294	ND
M1126	24	F	0·558	233	ND
M1141	24	F	0·850	364	ND
M929	25	F	0·984	186	ND

* Antibody level in pre-vaccine sample diluted 1 : 100; O.D. = optical density. There was no significant correlation of pre-vaccine antibody level and vaccine response either *in vivo* ($R = -0{\cdot}09$) or *in vitro* ($R = 0{\cdot}41$).

† Estimated by calculation of the area between pre- and post-vaccine serum dilutions (ELISA) as depicted in Fig. 1A.

‡ Estimated by calculation of pre- and post-vaccine cell dilution curves (MASELA) as depicted in Fig. 1B.

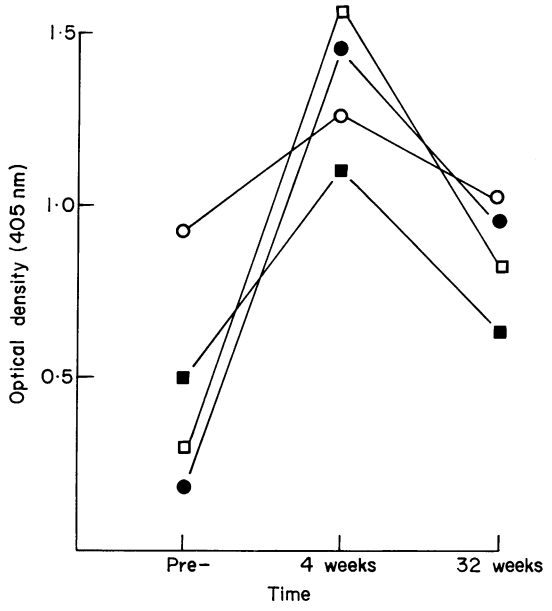

Fig. 2. Persistence of anti-influenza antibody. The antibody level (by ELISA) of four animals was determined both at 4 weeks and 32 weeks and in each case there was a fall off in antibody level by 32 weeks, but in three animals the level did not reach baseline. Monkeys: (●) AE 179; (□) 80035; (■) 80015; (○) 79151.

the cells were aspirated onto glass filter paper (MASH harvester) and the amount of radiolabel incorporated into DNA was determined by β-counter (Packard Tricarb 300 CD). Results were reported as net ct/min (experimental − background).

Natural killer (NK) cell activity. NK cell activity was determined by chromium release assay. For this 5×10^6 target cells (K562 human leukaemia cell line) were incubated with 125 μCi of sodium chromate (New England Nuclear, Boston, MA) in 0·05 ml media for 1 h at 37°C. The cells were then washed twice and suspended to 4×10^4 cells/ml in media containing 15% heat inactivated pooled human serum. Cytotoxicity was determined by measuring the release of the radiolabel by 2×10^3 target cells/well in the culture supernatant after 5 h of co-culture with MNC at effector:target ratios of 100:1, 33:1 and 11:1.

RESULTS

Influenza vaccine response

Like the vaccine response in humans, the response in monkeys was quite variable (Table 1). Two monkeys had no increase in measurable antibody after vaccine, and several others had only a modest increase. The content of antibody in the pre-vaccine samples was variable, perhaps reflecting natural experience with similar antigens. Nevertheless, pre-vaccine level did not correlate wth antibody response (Table 1). Because there was no correlation of pre-existing antibody and subsequent response we considered the initial measured antibody more likely resultant from cross-reactive or non-specific antibodies than reflective of prior exposure to these viral antigens. Age did not appear to be a factor in the level of response. In humans, duration of antibody level above baseline (pre-vaccine level) is approximately 6 months, with shorter durations observed in the elderly (Levine *et al.*, 1987). In the four monkeys that we were able to test several months after vaccine the level was above baseline in each, although there had been some decline from the level at 4 weeks (Fig. 2).

In vitro *Lymphocyte functions*

We have previously demonstrated in a relatively large survey of rhesus monkeys a range of reponses for the *in vitro* parameters employed in this study (Ershler *et al.*, 1988). The responses and variability in the current sample were similar to those observed in the larger survey and were also similar to those observed in human surveys. In this regard a few generalizations from both the monkey and human observations can be made. Response to each of the mitogens decline with advancing age, with the most striking differences in PHA and Con A, and less in PWM response. Of note we did observe reduced NK activity in the older monkeys, a finding less consistently reported in humans. Krishnaraj & Blanford, 1984). The age-related decline in mitogen response, but not NK cell activity, was greater in males. In the current study, however, only healthy females (of all ages) were analysed. What is apparent is that there was no significant correlation between response to any of these *in vitro* parameters of immune competence and vaccine response (Fig. 3). The correlation coefficient (R) for antibody response and PHA response was −0·55; con A response, −0·31; PWM response, 0·31; and NK activity. 0·05.

In vitro *anti-influenza antibody synthesis*

Six days after vaccine, lymphocytes were cultured in antigen-coated microtitre wells and specific antibody synthesized and secreted into the culture media was determined by ELISA. Unlike the other *in vitro* parameters mentioned above, there was an excellent correlation of this measure and the vaccine response as determined by serum antibody level (Fig. 4).

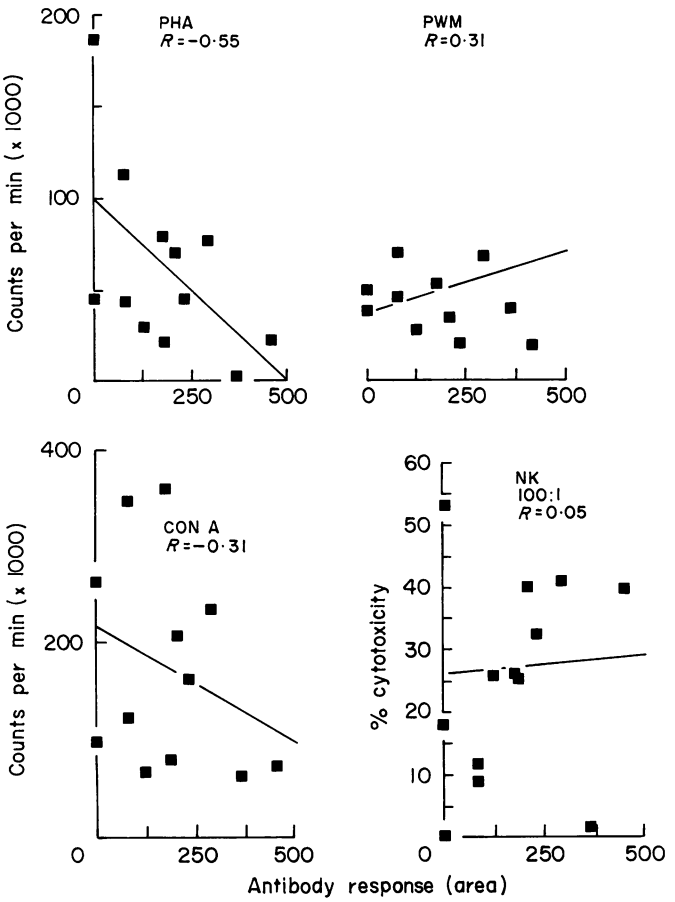

Fig. 3. Lymphocyte function correlation with antibody response. Lymphocyte cultures were stimulated with PHA, PWM or Con A or set up with radiolabelled targets in an NK assay and responses were recorded. As can be seen, there was a wide range of responses in these monkeys and there was no significant correlation with antibody response.

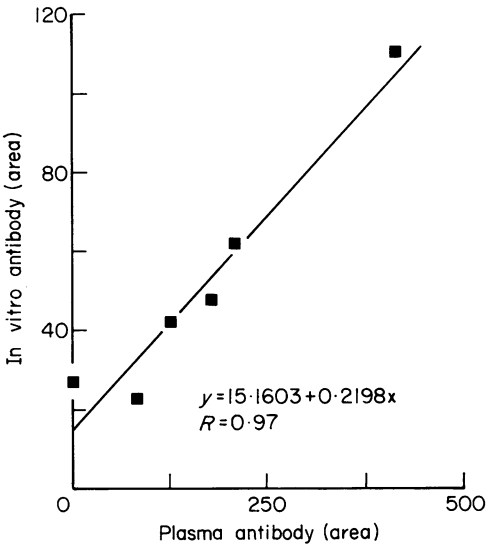

Fig. 4. *In vivo* and *in vitro* correlation. In six animals *in vitro* antibody synthesis was measured and compared to *in vivo* response to vaccine. As can be seen there was an excellent correlation of *in vitro* and *in vivo* response.

DISCUSSION

Clinical immunologists have developed a panel of *in vitro* tests of immune function that have proven to be of some utility in the assessment of immune competence. Accordingly, parameters such as lymphocyte proliferation in response to test mitogen, cytotoxicity or skin test reactivity, have been shown to be reduced in immune deficiency states such as Hodgkin's disease (Hillinger & Herzig, 1977; Weitzman *et al.*, 1977), corticosteroid treatment (Fauci, Dale & Balow, 1976; Hahn *et al.*, 1980) or aging (Walford, 1974; Weksler, Innes & Goldstein, 1976; Makinodan & Kay, 1980). The measures mentioned, however, are non-specific and are not truly predictive of response to a specific challenge. In contrast, determination of the specific antibody response to vaccine can be a very useful measure of immune capabilities in a well-defined system. The antibody response to protein antigens, such as influenza haemagglutinin, requires antigen presentation and complex cellular interactions in addition to the specific protein synthesis and secretion (Virelizier *et al.*, 1974), and a brisk response after vaccine is one reasonably good indicator that immune function is intact. Nevertheless, there are also complex genetic factors that regulate immune responsiveness, and antibody responses are highly variable among normal and healthy individuals (Paul, 1984). Vaccine response, therefore, is not a useful screen of immune competence.

The ability to measure specific antibody produced in lymphocyte cultures, however, could be of both clinical and research importance. Such a system might be useful in dissecting those cellular interactions involved in the antibody response and also be predictive of responders or non-responders to vaccine. In earlier work in our laboratory it was shown that lymphocytes from volunteers vaccinated 6 days prior to sampling produced measureable amounts of specific antibody when cultured. A combination of B and T cells were required for optimal response and viable cells, capable of protein synthesis, were essential (Moore, Ershler & Hacker, 1984). Furthermore, as expected, the presence of plastic-bound antigen did not appear to influence lymphocyte response (i.e., antibody level in the culture supernatant was equivalent in cultures established in antigen-coated wells or uncoated wells in which the supernatant was aspirated and tested separately for anti-influenza antibody). In those studies in humans we had also demonstrated that plasma antibody response after vaccine correlated well with *in vitro* production (Ershler, *et al.*, 1984).

In the current study we demonstrated in rhesus monkeys, like humans, that there is a wide range of lymphocyte reactivity in the commonly employed *in vitro* measures of blastogenesis and cytotoxicity, and furthermore, these lymphocyte functions appeared to correlate. Plasma antibody response after vaccine was also variable among individuals but no correlation was observed between antibody response and the lymphocyte assays (Fig. 2). In contrast, plasma antibody response was found to correlate highly with *in vitro* specific antibody synthesis measured in the MASELA. This correlation implies that the technique may be a predictor of vaccine response and also serve as a useful probe of the cellular and humoral interactions that are involved in antibody response. Furthermore, mechanisms of deficient antibody responses might be unravelled and immunopharmacologic techniques to override the defect might be tested

(e.g. Interleukin-2, thymic hormone or other biologic response modifiers). Such *in vitro* testing may prove useful in pre-clinical studies of such immuno-restorative agents.

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