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Mechanisms of protective immunogenicity of microbial vaccines: effects of cyclophosphamide pretreatment in Venezuelan encephalitis, Q fever and tularaemia

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SUMMARY

Administration of high-dose (250 mg/kg) cyclophosphamide (CY) to guinea-pigs and mice 3 days prior to immunization with inactivated vaccines derived from Venezuelan encephalitis virus (VE), *Coxiella burnetii* and *Francisella tularensis* resulted in accentuated and prolonged delayed-type hypersensitivity (DTH) and *in vitro* cellular immunity (CMI) to specific antigen. Humoral antibodies were either absent or significantly lower in CY-pretreated animals compared to immunized non-pretreated controls. CY pretreatments precluded protection in the VE virus model, suggesting that resistance is related to antibody. In the Q fever model, the protective immunogenicity of vaccine was preserved or increased by CY pretreatment suggesting that cell-mediated immunity is the important factor. In the tularaemia bacterial system, there was a complex effect of CY pretreatment on the low-grade protection afforded by killed vaccine against virulent infection. These findings suggest that the inability of killed vaccines to induce high-grade resistance against tularaemia and Q fever may be due in part to a suppressive B cell response which is eliminated by CY. These studies have given useful information on the relative significance of components of the specific immune response and may lead to an increased understanding of the mechanisms of action of vaccines and adjuvants.

INTRODUCTION

The cytotoxic drug, cyclophosphamide (CY), has variable effects on the immune response depending on the dosage and timing of administration with respect to immunization (Kerckhaert, Hofhuis & Willers, 1977a). In general, CY given in a single large dose at the time of immunization or after has been associated with suppression of both humoral and cellular components of the immune responses. High-dose CY given before immunization has the differential effect of suppressing humoral responses and either enhancing or not affecting cellular immune response. This has resulted in the character of skin reactions changing from Jones–Mote hypersensitivity to a more classical delayed-type hypersensitivity (DTH) reaction (Katz, Parker & Turk, 1975a). This latter effect is reportedly due to the elimination of a population of suppressor cells with surface characteristics of B cells (Katz, Parker & Turk, 1975b). Our previous study has shown the ability of such CY pretreatment to enhance guinea-pig delayed skin reactivity to tularaemia antigen emulsified in Freund's incomplete adjuvant (FIA) (Ascher, Parker & Turk, 1977). These studies have now been

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extended into animal challenge models designed to test the biological significance of the selective immunostimulation and immunosuppression produced by CY pretreatment. We have used three model infections: tularaemia, Q fever and Venezuelan encephalitis (VE) virus. The use of CY in combination with these infectious challenge models has allowed us to assess directly qualitative and quantitative relationships between the immune response and protective resistance.

MATERIALS AND METHODS

Animals. Outbred Hartley strain guinea-pigs of either sex, weighing 450–500 g were used. The animals were purchased from Buckberg Laboratory Animals (Tomkins Cove, NY) or obtained from the breeding colony of the Walter Reed Army Institute of Research, (Washington, DC). They were fed commercial guinea-pig chow and kale *ad libitum*. Outbred MAI:Swiss mice of either sex were obtained at 6 weeks of age from Microbiological Associates (Walkersville, Maryland). Male AKR/J mice were obtained at 6 weeks of age from the Jackson Laboratory (Bar Harbor, Maine). Mice were used at 11–15 weeks of age and were fed commercial mouse chow and water *ad libitum*.

Vaccine antigens. The inactivated tularaemia vaccine was prepared for immunization, skin testing and for additions to lymphocyte cultures as reported previously (Ascher *et al.*, 1977). The live vaccine strain tularaemia (LVS) was obtained from the Institute's stocks (Eigelsbach & Downs, 1961). The Q fever vaccine consisted of purified formalin-inactivated particulate Henzerling strain phase I *Coxiella burnetii* (Kishimoto *et al.*, 1978a). The VE vaccine was a formalin-inactivated tissue culture preparation certified for human use. Details of its development and clinical evaluation are published elsewhere (Cole, May & Eddy, 1974; Edelman *et al.*, 1979). The VE antigen for skin test and *in vitro* use was ultraviolet-inactivated TC-83 vaccine virus prepared as previously described (Marker & Ascher, 1976).

CY treatment. CY was purchased from Mead Johnson Company (Evansville, Indiana). It was dissolved in sterile water for injection, USP, and administered to mice and guinea-pigs as an intraperitoneal (i.p.) injection of 250 mg/kg 3 days prior to immunization.

Immunizations. The vaccines were diluted as noted in individual experimental results and emulsified with either an equal volume of Freund's complete adjuvant (FCA; DIFCO Laboratories, Detroit, Michigan) or Freund's incomplete adjuvant (FIA; DIFCO). Guinea-pigs received a total of 0·4 ml of emulsion as four equally divided doses, one into each footpad. Mice received injections of 0·1 ml of emulsion subcutaneously (s.c.). In some cases vaccines were administered without adjuvant. The LVS tularaemia vaccine was administered s.c. to mice in a dose of 10^2 organisms in 0·1 ml.

Skin testing. Guinea-pigs were skin tested by intradermal (i.d.) injection of the appropriate antigen into the shaved flank. Reactions were assessed by three parameters: the intensity and diameter of erythema and the increase in skin thickness using skin calipers (Schnelltaster, A02T, Kroplin, West Germany). Skin reactions were read at 4, 24, 48, 72 and 96 hr. The results of thickness measurements are expressed as the specific increase in skin thickness, which represents the reading (0·1 mm) at the skin test site minus the average thickness of normal skin on both sides of the site. Mouse DTH testing was accomplished by injecting 50 μl of antigen suspension into one rear footpad and measuring the footpad thickness at 24 hr with calipers. The results are expressed as the difference in thickness between the injected footpad and the control uninjected footpad (0·1 mm).

Lymphocyte transformation (LT) test. A whole-blood microculture technique for guinea-pig LT was employed as previously reported (Ascher *et al.*, 1977). Data are presented as the geometric mean incorporation of ^{14}C-thymidine of six replicate cultures and as the ratio of counts in antigen-containing cultures to that of controls (stimulation index, SI).

Serologic procedures. Antibody to VE was assayed by the protein A radioimmunoassay (Jahrling, Hesse & Metzger, 1978). Data are expressed as the titre of serum producing 50% precipitation of labelled virus. Plaque reduction neutralization (PRN) tests to VE were performed as previously described (Jahrling *et al.*, 1978). In Q fever-immunized guinea-pigs, serum antibody activity was determined by the indirect immunofluorescent antibody technique against phase II *C. burnetii* (Bozeman & Elisberg, 1963).

Challenge studies. (i) In VE studies, mice were injected i.p. with 0·3 ml of a Trinidad donkey VE virus suspension containing approximately 7×10^5 plaque-forming units (PFU). Guinea-pigs were injected s.c. with 0·2 ml of VE Trinidad donkey virus suspension containing 8×10^3 PFU and animals were observed daily for death.

(ii) For tularaemia challenge, mice were injected i.p. with 0·2 ml saline containing either 10^2 SCHU S4 or 425 organisms verified by viable cell count of the inoculum and observed for death.

(iii) Guinea-pigs were challenged with an aerosol of 10^4 minimal infective doses of phase I Henzerling Strain *C. burnetii* as previously described (Kishimoto & Burger, 1977). Rectal temperatures were taken twice daily; two consecutive temperature readings of 40°C or greater were considered evidence of infection.

Experimental design. All animals were given CY 3 days prior to immunization, and bled on day 11 for serologic and LT studies. Skin testing or challenge was performed on day 14. Guinea-pigs were studied in groups of five or six and mice in groups of ten or more.

Statistical analyses. Single comparisons were made with Student's *t*-test and multiple comparisons with the one-way analysis of variance. The protective dose of 50% of animals (PD_{50}) was calculated and comparisons were made with the Spearman–Karber method (Finney, 1952). Comparisons of survival percentages in challenge experiments were made with the four-fold table test (Diem, 1962).

RESULTS

Effect of CY pretreatment on the immune response to VE vaccine

Three types of immune responses to VE were measured in guinea-pigs after administration of inactivated vaccine. Delayed skin reactivity to VE viral antigen was assessed 14 days after immunization. As a control for the skin test, a dose of 0·1 μg protein elicited a very low level of non-specific skin reactivity and no immunity in non-immune controls (data not shown). In immunized animals (Table 1), there was preservation of DTH reactivity after CY pretreatment when either diameter of erythema or skin thickness changes were considered. As an *in vitro* correlate of CMI, lymphocyte transformation to VE viral antigen was measured in a similar group of animals 11 days after immunization. There was a significant stimulation of lymphocytes only in those guinea-pigs pretreated with CY (Table 2) ($P < 0·05$ by analysis of variance). This has been a consistent finding in this viral system. In sharp contrast, the serologic data shown in Table 3 show a total abolition of humoral antibody to VE at 11 days brought about by CY pretreatment measured by either neutralization of virus or radioimmunoassay ($P < 0·05$).

Table 1. Effect of cyclophosphamide pretreatment on delayed-type hypersensitivity to VE antigen in guinea-pigs (six/group)

		Mean increase in skin thickness (mm)		
Immunization	CY (mg/kg)	4 hr	24 hr	48 hr
None	0	0·67 ± 0·21† (0)	0·00 ± 0·16 (0)	0·00 ± 0·42 (0)
VE-FIA*	0	0·50 ± 0·43 (0)	4·40 ± 0·61 (8)	2·17 ± 0·54 (5)
VE-FIA	250	1·08 ± 0·35 (0)	4·17 ± 0·61 (9)	2·17 ± 0·46 (6)

* C-84 vaccine diluted 1:6 with saline and emulsified with an equal part of FIA.

† Results expressed as mean ± s.e.m. Figures in parentheses indicate diameter.

Table 2. Effect of cyclophosphamide pretreatment on LT responses of guinea-pigs to VE antigen

Immunization	CY (mg/kg)	*n*	Geometric mean c.p.m. well (SI) None	VEE*
None	0	3	56	59 (1·07)
VE-FIA†	0	6	70	115 (1·78)
VE-FIA	250	5	63	198 (3·24)

* Purified VE viral antigen, 1 μg/ml.

† C-84 vaccine diluted 1:6 with saline and emulsified in FIA.

Table 3. Effect of cyclophosphamide pretreatment on geometric mean humoral antibody response of guinea-pigs (five/group) to VE virus vaccine

Immunization	CY (mg/kg)	Radioimmunoassay$_{50}$ (range)	PRN_{80} (range)
None	0	< 80 (—)	< 10 (—)
VE-FIA*	0	246 (160–709)	17 (< 10–80)
VE-FIA	250	< 80 (—)	< 10 (—)

* C-84 vaccine diluted 1:1 with saline and emulsified with an equal part of FIA.

Animals prepared according to the same procedures were challenged s.c. with VE at 14 days after immunization. In three successive preliminary experiments using undiluted vaccine in FIA (data not shown), survival of immunized animals pretreated with CY was much lower than that of immunized controls but controls were not consistently protected. In two other experiments (data not shown), one using undiluted vaccine with challenge at day 14 and one with a later challenge at day 21 using vaccine diluted 1:6, all animals were protected. To assess more fully the effects of CY, we then eliminated the Freund's adjuvant and used several concentrations of vaccine. In such a dose–response study demonstrated in Fig. 1, there was a marked decrease in survival due to CY pretreatment ($P < 0{\cdot}02$ by four-fold table test). A similar experiment studying CY effects across a dose range of vaccine conducted in AKR/J mice is illustrated in Fig. 2. Again, a marked decrease in protection ($P < 0{\cdot}001$) is noted. We therefore conclude that the effects of CY are readily apparent under conditions of marginal protection but can be overcome by too large a dose of vaccine or by waiting a longer interval (21 days) between immunization and challenge. In another experiment (data not shown), guinea-pigs were subjected to an i.d. challenge of live VE. DTH reactivity was then measured along with survival. The skin reactions are similar to those seen in Table 1 and the survival shown in Fig. 1. Thus, intentionally biasing the system in favour of the action of DTH to injected virus by using an i.d. route of inoculation did not appear to change the outcome of the experiment.

Effect of CY pretreatment on the immune response to Q fever vaccine

A similar group of measurements was performed in guinea-pigs following immunization with the Q fever vaccine. The skin test response presented as change in thickness of immunized guinea-pigs with and without CY pretreatment is presented in Fig. 3. The increase due to CY persists through

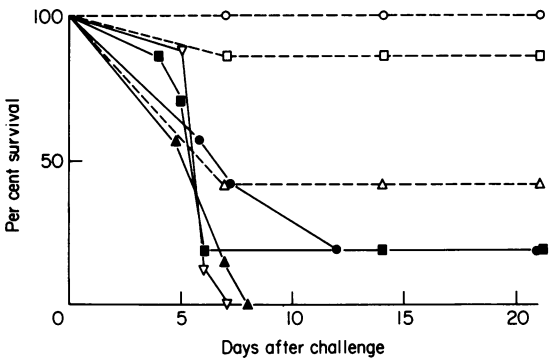

Fig. 1. Effects of CY pretreatment on protective immunogenicity of VE vaccine in guinea-pigs challenged 14 days after vaccine (seven/group). (▽——▽) Controls; (○- - -○) VEE 1:5, (●——●)+CY; (□- - -□) VEE 1:15, (■——■)+CY; (△- - -△) VEE 1:50, (▲——▲)+CY.

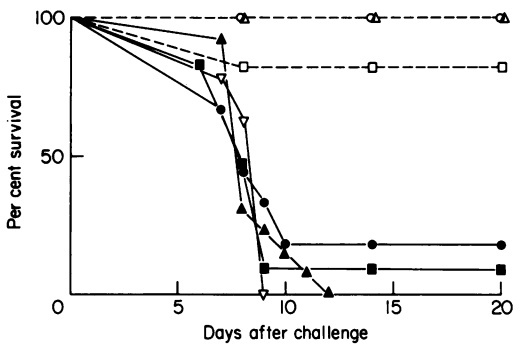

Fig. 2. Effects of CY pretreatment on protective immunogenicity of VE virus vaccine in mice challenged 14 days after vaccine (nine to twelve/group). (▽——▽) Controls; (○- - -○) VEE 1:2, (●——●)+CY; (□——□) VEE 1:4, (■——■)+CY; (△- - -△) VEE 1:8, (▲——▲)+CY.

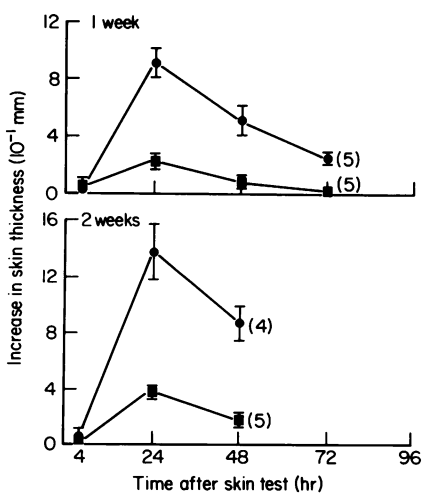

Fig. 3. Effects of CY pretreatment on guinea-pig delayed hypersensitivity to Q fever antigen 1 and 2 weeks after immunization (four to five/group). (■——■) Q-FIA, (●——●) CY-Q-FIA.

Table 4. Effect of cyclophosphamide pretreatment on LT responses of guinea-pigs (three/group) 11 days after Q fever vaccination

Immunization	CY (mg/kg)	Geometric mean c.p.m./well (SI) None	Phase I*	Phase II*
None	0	84	103 (1·27)	58 (0·7)
Skin test only	0	143	180 (1·27)	108 (0·8)
Q-FIA†	0	113	169 (1·70)	128 (1·50)
Q-FIA	250	134	727 (5·60)	644 (5·07)

* 1:1,000 final concentration.
† 12 μg phase I Q fever vaccine emulsified with FIA.

Table 5. Effect of cyclophosphamide pretreatment on the humoral immune response to phase II Q fever antigen

Vaccine (μg)	CY (mg/kg)	No. positive/total by IFA titre 1:10	1:50
3	0	3/3	3/3
3	250	1/3	0/3

the second week after immunization ($P < 0·05$). The time course of this increase had been shown to be a function of the dose of immunizing antigen in our previous study (Ascher *et al.*, 1977).

The lymphocyte transformation data of similarly prepared guinea-pigs were measured on day 11. As can be seen in Table 4, CY pretreatment is associated with an increase in reactivity to both phase I and II Q antigens without a significant change in background counts ($P < 0·01$). These data are similar to those reported previously in the tularaemia model (Ascher *et al.*, 1977).

The antibody response of similar groups of guinea-pigs is shown in Table 5. There is a marked decrease if not a total abolition of antibody resulting from CY pretreatment ($P < 0·05$). These data are qualitatively similar to those presented for VE.

The effect of CY pretreatment on protection against Q fever was also determined. The dose–response protection data with phase I Q fever vaccine with and without FIA is shown in Table 6. Fever was used as the end-point since Q fever is not lethal for guinea-pigs under these conditions. In the presence or absence of FIA, the protection of animals is not significantly affected by CY when the calculated protective doses for 50% of the animals (PD_{50}) are compared.

Effect of CY pretreatment on the immune response to tularaemia vaccine

The findings previously published for guinea-pigs with a tularaemia model have now been extended to mice. The effect of CY pretreatment on mouse DTH to specific antigen measured by the footpad swelling test is presented in Fig. 4. There is very little effect of adjuvants on potentiating DTH in this system in the absence of CY. The increase in DTH reactions brought about by CY approximates that seen in recipients of live vaccine ($P < 0·05$).

Mice prepared in this manner were then challenged with virulent tularaemia. Two different challenge strains were employed, an 'A' strain organism, SCHU S4 and a 'B' strain organism, 425. The result of CY pretreatment of the protection afforded by killed vaccine against SCHU is seen in Fig. 5. There is very little effect on the survival of mice due to killed vaccine alone or in combination with either adjuvant. Pretreatment with CY is also ineffective in altering the inexorable lethal

Table 6. Effect of cyclophosphamide pretreatment on protective immunogenicity of Q fever phase I vaccine with and without Freund's incomplete adjuvant

Immunizing dose (μg)	No. febrile/total (PD_{50}) None	Cytoxan (250 mg/kg i.p.)	
With FIA			
0	5/5	6/6	
0·03	3/3	4/5	
			(0·15)
0·3	4/5	2/5	
	(0·60)		
3·0	0/5	0/5	
Without FIA			
0	6/6	n.d.	
0·25	5/6	5/5	
0·5	6/6	5/6	
1·0	4/6	4/6	
	(1·40)		(1·40)
2·0	3/6	3/6	

n.d. = Not done.

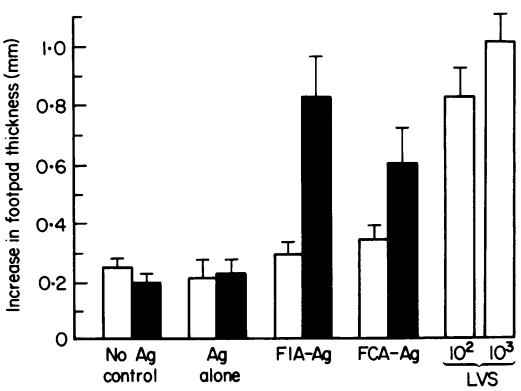

Fig. 4. Effects of CY pretreatment on mouse delayed hypersensitivity to tularaemia vaccine 18 days after immunization (nine to twelve/group). (□) No cytoxan, (■) cytoxan.

outcome of SCHU S4 infection. These results are in sharp contrast to the high-grade resistance manifested as universal long-term survival against similar challenge by recipients of LVS vaccine (data not shown).

The opportunity exists with tularaemia to use another strain of organism with less virulence for man and experimental animals. There is a slightly better degree of protection noted with killed vaccine in these challenge systems than in the SCHU model. Fig. 6 shows the result of varying doses of CY pretreatment on the protective immunogenicity of killed tularaemia vaccine in combination with FIA. We note a stepwise increase in survival in CY recipients related to the dose until the 250 mg/kg dose at which point the protection of killed vaccine is totally abrogated.

DISCUSSION

There are numerous studies on the effects of CY in the course of infection which show potentiation

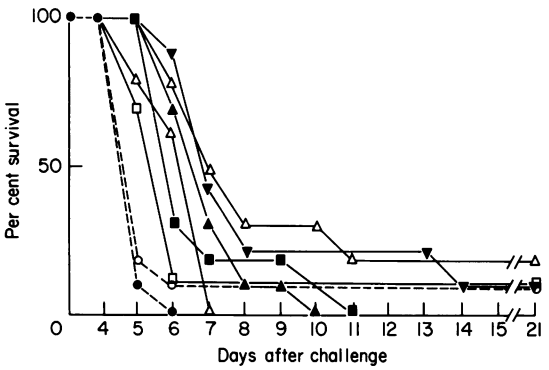

Fig. 5. Effects of CY pretreatment on protective immunogenicity of killed tularaemia vaccine in mice challenged days after immunization (ten to twelve/group). (●– – –●) Control, (○– – –○) + cytoxan pretreatment; (■——■) antigen, (□——□) + cytoxan pretreatment; (▲——▲) FIA-Ag, (△——△)+ cytoxan pretreatment; (▼——▼) FCA-AG, (▽——▽)+cytoxan pretreatment.

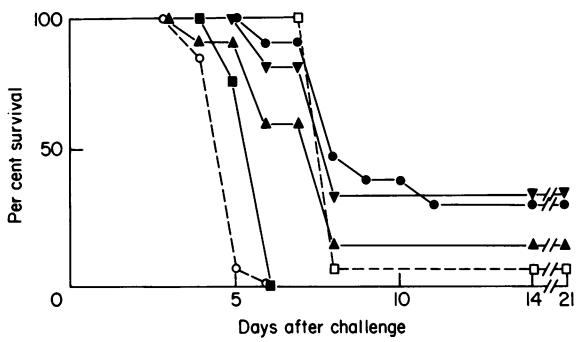

Fig. 6. Effect of increasing doses of CY pretreatment on the protective immunogenicity of killed tularaemia vaccine in mice challenged 21 days after immunization (nine to twelve/group). (○– – –○) Control, (□- - -□) FIA-TUL, (▲——▲)+16 mg/kg CY, (▼——▼)+40 mg/kg CY, (●——●)+100 mg/kg CY, (■——■)+250 mg/kg CY.

of viral (Jahrling, Dendy & Eddy, 1974; Miller *et al.*, 1978), rickettsial (Tachibana & Kobayashi, 1975), bacterial (Tripathy & Mackaness, 1969; Pierson, Johnson & Feller, 1976), fungal (Cozad & Lindsey, 1974; Beaman & Maslan, 1977; Graybill & Mitchell, 1978), mycobacterial (Shepard & Redus, 1967) or parasitic diseases (Finerty & Krehl, 1976; Belehu, Poulter & Turk, 1976; Modabber, Bear & Cerny, 1976). However, there are a few studies in which CY is given prior to a single immunization with an inactivated vaccine followed by an infectious challenge after an appropriate interval. Studies, which on the surface are similar to this design, have been reported but differed significantly in the use of multiple doses of CY or vaccine (Weiner, Cole & Nathanson, 1971; Wells, Diggs & Phillips, 1977). The emphasis on the inactivated nature of the vaccine is that if CY is given before live vaccine (Kerckhaert, Hofhuis & Willers, 1977b), it has effects on primary infection by the vaccine in addition to effects on the immune system. This complication has not always been taken into account in published reports. Our studies take advantage of the well described selective immunosuppression brought about by single-dose CY pretreatment to define the protective component of the specific immune response.

The mechanisms of protection in VE are not fully understood. Immune serum affords a high degree of protection to experimental animals (Berge *et al.*, 1961). Studies of VE infection in nude mice have shown potentiation of infection (Leblanc, Scherer & Sussdorf, 1978) and pretreatment with anti-thymocyte serum has been reported to result in lessened infection (Woodman, McManus & Eddy, 1975). Other workers using cellular transfer have implicated both an immunocyte and antibody in protection of mice against VE (Rabiñowitz & Adler, 1973; Rabinowitz, 1976a, 1976b).

However, evidence for the nature of the cell is confusing. Studies in other viral systems concerning the relative significance of humoral and cellular immunity have also shown mixed results (Araullo-Cruz, Ho & Armstrong, 1978; Oakes, & Rosemond-Hornbeak, 1978). Our results indicate that antibody is the major factor in active immunity induced by VE vaccine. This is based on the susceptibility of CY-pretreated antibody-negative animals to VE infection in the presence of intact DTH and cellular immune reactivity to specific antigen. In contrast to a recent report (LaGrange *et al.*, 1978), we have been able to demonstrate unaltered DTH to a viral antigen after CY pretreatment. In the experiment in which i.d. challenge was used, DTH reactivity was equivalent in intensity regardless of CY pretreatment, but the outcome was lethal for those animals without antibody. There can be little doubt that the latter animals recognized VE antigen but the type of split immune response manifested was ineffective in limiting the infection.

The mechanisms of protective immunity in Q fever have not been fully elucidated to date. Early workers noted inhibition of rickettsial growth when certain immune sera were incubated with challenge organisms prior to inoculation (Abinanti & Marmion, 1957). In later work, antibody has been implicated as a co-factor in the handling of *C. burnetii* by immune macrophages (Kishimoto & Walker, 1976; Kishimoto *et al.*, 1977). Factors in supernatants from stimulated lymphocyte cultures assist in macrophage resistance in a similar *in vitro* test system (Hinrichs & Jerrells, 1976). Subsequently, several reports have noted cellular immune reactivity in patients and animals convalescent from Q fever (Jerrells, Mallavia & Hinrichs, 1975) and animal recipients of inactivated vaccine (Kishimoto *et al.*, 1978a). Q fever infection of nude mice results in a prolonged illness without mortality (Kishimoto, Rozmiarek & Larson, 1978b). A role for specific T cell-mediated immune responses in protection has been established by direct cell transfer experiments in scrub typhus, another rickettsial system (Shirai *et al.*, 1976). This phenomenon was seen only with heterologous challenge; homologous protection could be demonstrated with immune serum (Catanzaro *et al.*, 1977). In our system, homologous protection is preserved in the face of a marked decrease in antibody titre after CY pretreatment and vaccine. One distinct advantage of CY pretreatment as a probe of the immune responses is the relative simplicity of CY experiments compared to cell transfer techniques. Nevertheless, the findings in this model should be verified by cell transfer experiments. The question of whether the CY effect results in an actual increase of protective immunogenicity of vaccine is a very important one. There are two possible explanations for such an effect. First, it is possible that antibody *per se* has a deleterious effect on protection in Q fever. Secondly and more likely, B cell suppression of CMI may be associated with a decrease of protection. If this could be established in transfer experiments, then a case could be made that Jones–Mote reactivity or its modern equivalent, cutaneous basophil hypersensitivity (CBH), may be an undesirable response to immunization. This is particularly interesting in that CBH is being reported in more and more situations (Dvorak, 1976). The approach we have described may lead to an increased understanding of the functional significance of CBH.

The tularaemia model is more complex. Several features of the history of tularaemia immunity are worth noting. High-grade protection of laboratory animals and at-risk personnel against virulent organisms was not accomplished with the killed vaccine used in these studies (Eigelsbach & Downs, 1961; Foshay *et al.*, 1942; Andron & Eigelsbach, 1975). A live attenuated strain of *F. tularensis* was developed which induced both high-grade DTH reactivity and resistance to challenge (Eigelsbach & Downs, 1961). The relative nature of the resistance in man has been emphasized in a recent retrospective survey (Burke, 1977). A lack of correlation between DTH and resistance in tularaemia has been reported on the basis of experiments showing no ill effects of desensitization of DTH on protection (Gordon, 1963). Similarly, immunity in the absence of DTH has been reported in listeriosis (Osebold, Pearson & Medin, 1974). In tularaemia, cellular transfer experiments have shown mixed results depending on the end-point (Allen, 1962; Eigelsbach *et al.*, 1975); there are a number of reports on vaccines showing variable effects on different challenges in several species of animals (Larson, Bell & Owen, 1954; Bell *et al.*, 1952; Woodward *et al.*, 1964) There is, however, no information available on the use of adjuvants with killed vaccines or employing immunomodulation such as that produced by CY or both. In studies with the virulent challenge organism SCHU S4, we have been unable to produce significant protection with killed vaccine. It would appear that the mechanism of protection afforded in this model by live vaccine is non-specific macrophage

activation although published results have not made that clear (Claflin & Larson, 1972; Kostiala, McGregor & Logic, 1975).

In studies employing as challenge the strain 425 of *F. tularensis*, we considered it important to assess the interaction between serum antibody and CMI as seen in a Brucella model recently reported (Bascoul *et al.*, 1978). We therefore elected to use lower doses of CY for pretreatment in the hope that, as reported by others (Askenase, Hayden & Gershon, 1975), a dose of CY could be found at which antibody response would be preserved along with enhanced CMI. Following this approach, we note improvement in survival with moderate doses of CY. We have not been technically able to detect antibody responses of mice after tularaemia vaccine, and are not able to define clearly the point at which antibody is completely eliminated. As in the case of Q fever, the inability of killed vaccine in the tularaemia system to afford mice high-grade protection may be due to the generation and action of suppressor B cells which interfere with, or limit the expression of, a protective specific cellular immune response. Our results make this hypothesis likely since the elimination of suppressor cells and the elevated DTH which results is associated with increased resistance. The possible predilection for generation of suppressor activity after immunization with bacterial products has been raised in a recent report (Colizzi *et al.*, 1978). The loss of protection after the 250 mg/kg dose is not unexpected with this organism and clearly shows interaction between humoral and cellular immunity. The slight stepwise increase in resistance in CY-pretreated immunized mice in this system is encouraging but meagre compared to the absolute resistance possessed by recipients of live vaccine. That these two groups of animals possess such great differences in resistance in the face of equivalent DTH reactivity is intriguing.

Our results demonstrate the utility of CY-induced simultaneous immunosuppression–immunostimulation to assess the relative role of the components of the immune response in resistance. The ability of cyclophosphamide to inhibit preferentially B cell function and antibody formation along with preservation or augmentation of cellular immune reactivity has allowed us to assess the relative importance of the two major arms of the specific immune response in three different model infections. The immunomodification produced by CY pretreatment is unique in that in most other cases of immunosuppressors or immunostimulators there is an effect of similar direction on all components of immune responsiveness. Further studies testing other candidate vaccines and adjuvant substances under the influence of CY pretreatment may give useful new information on how to design and most effectively administer vaccines in man.

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In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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