CHANGES IN SPLEEN AND THYMUS CELL PHENOTYPES IN MICE VACCINATED WITH THE *COXIELLA BURNETII* PHASE I WHOLE-CELL VACCINE OR THE CHLOROFORM-METHANOL RESIDUE SUBUNIT VACCINE

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Summary. – Lymphoid cell phenotypes within the spleen and thymus were analyzed to determine whether numerical or proportional changes in cell populations could account for the immunosuppression seen after vaccination of mice with inactivated phase I *Coxiella burnetii* whole-cell vaccine (WCI). Within 21 days of vaccination with WCI, there was a reduction in the percentage of splenic T cells and B cells while the numbers of thymic T cells and B cells increased. A substantial percentage of spleen cells did not bear typical T cell or B cell surface markers. In contrast, except for an early rise (by day 3) in the numbers of T and B cells, injecting the chloroform-methanol residue subunit vaccine (CMR) caused no significant phenotypic changes of lymphoid cells in the spleen or thymus. The percentage of thymus cells bearing T cell phenotypes was similar in mice vaccinated with WCI or CMR. However, the total number of T cells in the thymus dramatically decreased in mice vaccinated with WCI. There was no correlation between the lymphocyte hyporesponsiveness to mitogens and WCI in vitro and the increased numbers of CD8-positive splenocytes. These results suggest that WCI vaccination caused dramatic changes in splenocyte and thymocyte lymphocyte populations and provide evidence for the more benign nature of the CMR vaccine.

Key words: Q fever; vaccine; immunity; leukocyte phenotypes

Introduction

Coxiella burnetii, the etiological agent of Q fever, is an obligate intracellular bacterial pathogen of the eucaryotic phagolysosome. In response to infection by *C. burnetii*, the human host mounts a vigorous immune response that may eliminate the microorganism. A small percentage of persons with acute Q fever may develop chronic Q fever, characterized primarily by endocarditis (Raoult *et al.*, 1990). Suppression of the immune response may allow a *C. burnetii* infection to become chronic (Koster *et al.*, 1985). The mechanism of suppression in humans chronically infected with *C. burnetii* involves an antigen-specific suppressor T cell-monocyte circuit (Koster *et al.*, 1985). Although there are no satisfactory animal models to mimic the pathogenesis established in chronically infected humans, the lymphocytes of laboratory mice undergo immunosuppression after infection or vaccination with phase I *C. burnetii* (Damrow *et al.*, 1985). The suppression of thymidine uptake into murine lymphocytes exposed to *C. burnetii* antigens in culture is not antigen-specific; as responses to concanavalin A (ConA), phytohemaglutinin A (PHA), pokeweed

Abbreviations: CMR = phase I *C. burnetii* chloroform-methanol residue subunit vaccine; Con A = concanavalin A; ELISA = enzyme-linked immunosorbent assay; ip = intraperitoneal(ly); LPS = lipopolysaccharide; PBS = phosphate buffered saline; PHA = phytohaemagglutinin; PWM = pokeweed mitogen; WCI = inactivated phase I *C. burnetii* whole-cell (vaccine)

mitogen (PWM) and other heterologous antigens (Waag, D.M., unpublished results) are also suppressed.

Injecting mice with WCI causes hepatomegaly, splenomegaly, liver necrosis and death in a dose-dependent manner (Williams and Cantrell, 1982). Moreover, vaccinating previously immune humans with WCI can cause granulomas and sterile abscesses at the injection site (Benenson, 1959). In contrast to these deleterious effects, laboratory animals injected with WCI displayed enhanced non-specific resistance to tumors, and to protozoan, bacterial, and viral infections (Kelly *et al.*, 1976; Kelly, 1977; Clark, 1979; Kazár and Schramek, 1984; Waag *et al*, 1990). This dichotomy of responses may result from divergent responses of host cells to antigenic stimulation. Certain populations of lymphocytes may be stimulated while others are suppressed by WCI vaccination.

The latest generation Q fever vaccine is the residue (CMR) remaining after chloroform:methanol (4:1) extraction of WCI. Results suggest that the CMR vaccine is safe and immunogenic in animal models and humans and does not induce pathological reactions or suppression of splenocytes cultured *in vitro*, even when given at doses several times higher than WCI doses (Williams *et al.*, 1986). Moreover, CMR has been shown to enhance non-specific resistance to challenge with infectious agents (Zvilich *et al.*, 1995). Marked differences in the ability of WCI and CMR to cause adverse pathological reactions and lymphocyte hyporesponsiveness provided the impetus to study cell surface phenotypic changes in leukocytes from mice vaccinated with WCI or CMR.

Materials and Methods

Vaccines. Six-week-old C57BL/6J female mice (Harlan Sprague-Dawley, Indianapolis, IN) were injected intraperitoneally (ip) with 100 µg of WCI or CMR prepared in our laboratory from the *C. burnetii* phase I Nine Mile strain. WCI was grown in the yolk sacs of embryonated hen eggs and purified as previously described (Williams *et al.*, 1981). CMR was prepared from WCI according to the method of Williams and Cantrell (1982).

Antibody. Immunoglobulin concentrations in the sera of immunized mice were measured by enzyme-linked immunosorbent assay (ELISA) as described by Uhaa *et al.* (1994). Sera were diluted from 1:50 through 1:102,400. Sera with titers less than 50 were arbitrarily assigned a titer of 25 ($\log_2$ 4.64). Antibody titers against WCI, phase II whole-cell antigen (WCII), and phase I lipopolysaccharide (LPS) (Amano and Williams, 1984) were assayed by using an anti-mouse IgG (heavy and light chain) alkaline phosphatase conjugate. The intensity of the colorimetric reaction was assessed by reading absorbance at 405 and 630 nm (A_{405}, A_{630}). Titration endpoints were determined by noting the highest serum dilution with a minimum difference of 0.05 A unit between the non-antigen- and antigen-containing wells.

Cytological analysis. At 3, 7, 14 or 21 days after vaccination, mice (four per group) were deeply anesthesized with an ip injection of ketamine (200 mg/kg) and acepromazine (5 mg/kg) and euthanized by exsanguination. Spleens and thymuses from each group of mice were excised, pooled and dissociated into a single cell suspension, which was pelleted by centrifugation. The pellet was resuspended in 0.16 mol/l NH_4Cl with 0.1 mol/l Tris pH 7.4 (9:1) to lyse the red blood cells. One million spleen or thymus cells were washed with phosphate buffered saline (PBS) containing 1% bovine serum albumin and washed a second time with PBS containing 1% normal mouse serum to block Fc receptors. Cells were incubated with fluorescein-labelled monoclonal antibodies directed against specific cell-surface epitopes (indicated in brackets): CD4 (L3T4), CD8 (Lyt-2), Thy-1.2, B-220 (CD45R), class II major histocompatibility complex antigen (Ia), Ig (sIg) and CD45. Anti-CD4 identifies a differentiation antigen expressed on thymocytes and a subpopulation of mature T-helper cells; anti-CD8 defines the alpha chain differentiation antigen expressed on most thymocytes and a subpopulation of mature T cells of the cytotoxic-suppressor lineage; anti-Thy-1.2 labels 100% of T cells from mouse strains expressing the Thy-1.2 allele; anti-B-220 reacts with B-220 determinants expressed on pre-B and B lymphocytes; anti-Ia labels B cells, but not macrophages, in these experiments; anti-sIg labels cells bearing surface Ig; and anti-CD45 labels all leukocytes. Monoclonal antibodies were titrated against each cell sample to ensure the optimum staining of leukocytes. In a final step, cells were incubated with propidium iodide to stain dead cells and to exclude them from analysis. The processed cells were passed through a FACScan (Becton-Dickinson, Sunnyvale, CA) flow cytometer to determine the percentage of cells bearing each cell surface marker. The average number of labelled cells per mouse spleen or thymus was determined by multiplying the percentage of cells bearing each marker by the average number of nucleated spleen or thymus cells per vaccinated mouse. Day 0 (baseline) values represent the means of cytological analyses performed on spleens and thymuses 3, 7, 14 or 21 days after mice were injected ip with 0.5 ml of PBS.

Results

Antibody responses induced by vaccination with WCI or CMR

Antibodies were detected by ELISA within 7 days after vaccination with either WCI or CMR (Fig. 1). Titers increased through day 21. Antibody profiles of mice injected with either WCI or CMR were similar, although antibody responses after WCI vaccination were somewhat greater. After WCI or CMR vaccination, the WCII-specific antibody responses were consistently greater than the anti-WCI and anti-LPS responses.

Changes in the percentage of leukocytes bearing specific cell surface markers after vaccination of mice with WCI or CMR

In mice vaccinated with WCI, the percentage of nucleated spleen cells bearing B-cell markers (B-220, Ia, or sIg) remained at approximately 60% for 7 days after vaccina-

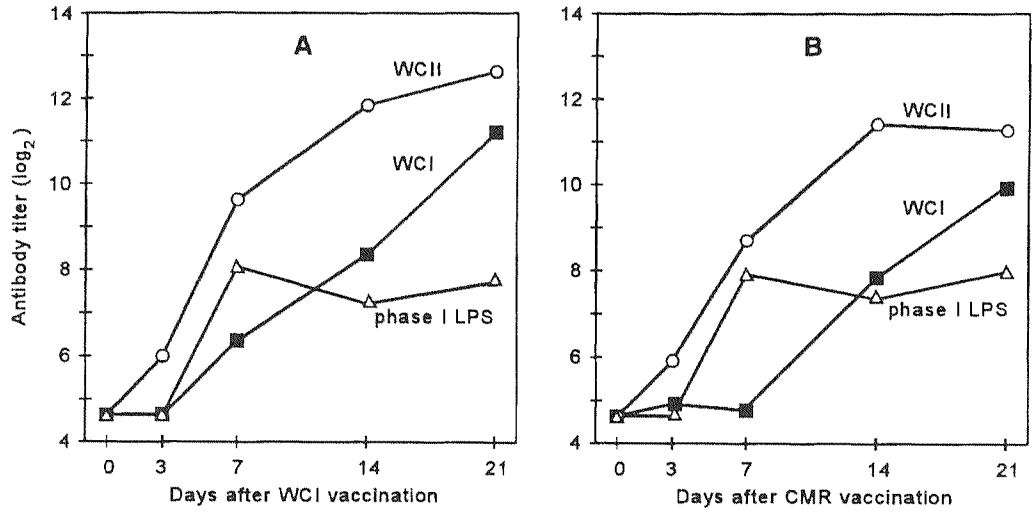

Fig. 1
Serum ELISA antibody titers of mice immunized with WCI (A) or CMR (B) vaccine

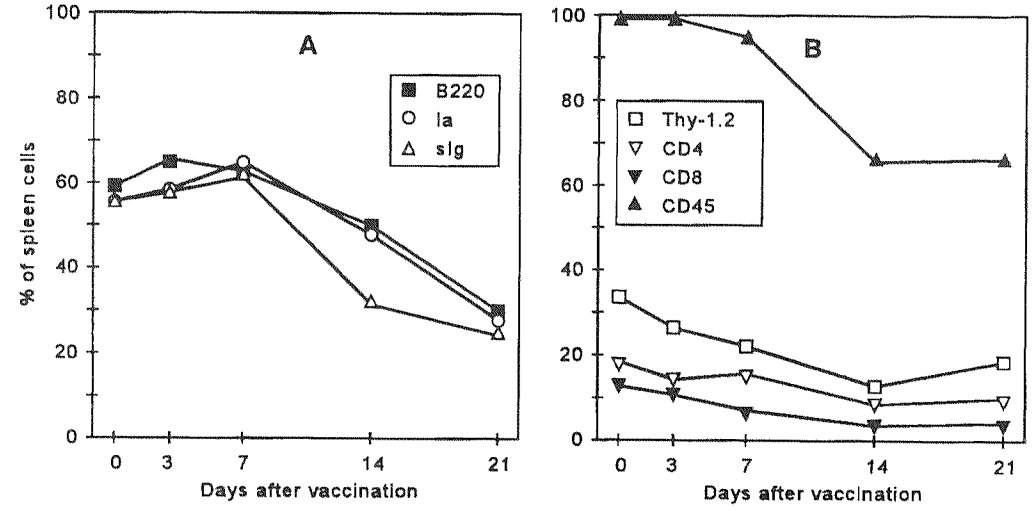

Fig. 2
Percentage of splenocytes from WCI-immunized mice bearing B cell (A), T cell or leukocyte (B) surface epitopes

tion and then declined to approximately 30% of nucleated spleen cells by 21 days after vaccination (Fig. 2A). The drop in sIg-bearing cells was somewhat more precipitous than the decline in the percentage of spleen cells bearing the B-220 or Ia surface markers.

In WCI-injected mice, the percentage of spleen cells bearing T-cell markers (CD4, CD8, or Thy-1.2) declined more gradually than the percentage of cells having B cell-specific surface markers (Fig. 2B). Within 14 days of vaccination, the percentage of splenocytes bearing the Thy-1.2, CD4 or CD8 markers declined from 33, 19, and 13 to 20, 10 and 5%, respectively. Interestingly, while the percentage of spleen cells bearing the CD45 marker was 100% before vaccination with WCI, the percentage of CD45-bearing cells decreased to 66% of nucleated spleen cells by day 14 after vaccination. Wright-stained smears prepared from these spleens did not reveal any unusual cell phenotypes (data not shown).

When vaccinated with CMR, the percentage of each spleen cell population assayed did not change through day 21 after vaccination (Fig. 3). In addition, all spleen cells were CD45-positive throughout the experiment.

The percentages of thymocytes bearing T cell surface markers of interest were very similar in mice vaccinated

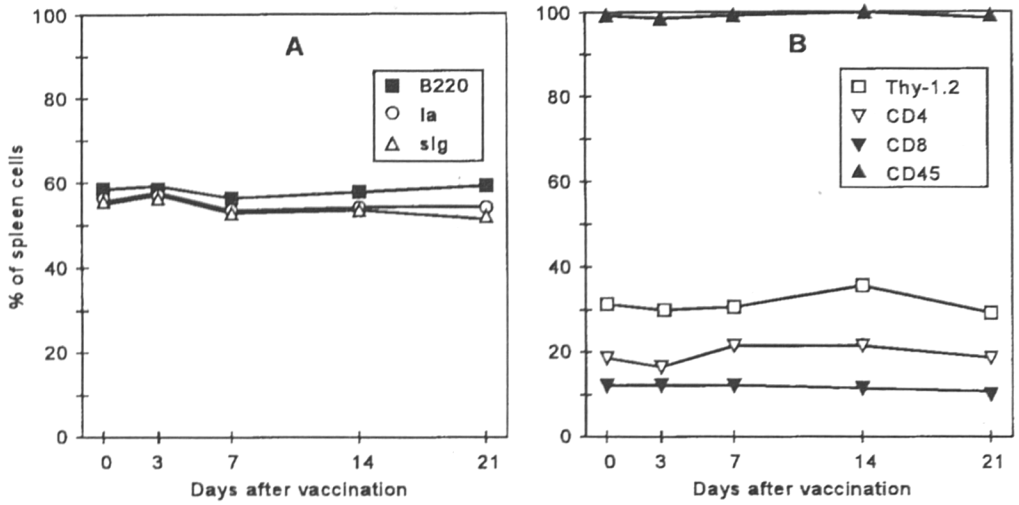

Fig. 3
Percentage of splenocytes from CMR-immunized mice bearing B cell (A), T cell or leukocyte (B) surface epitopes

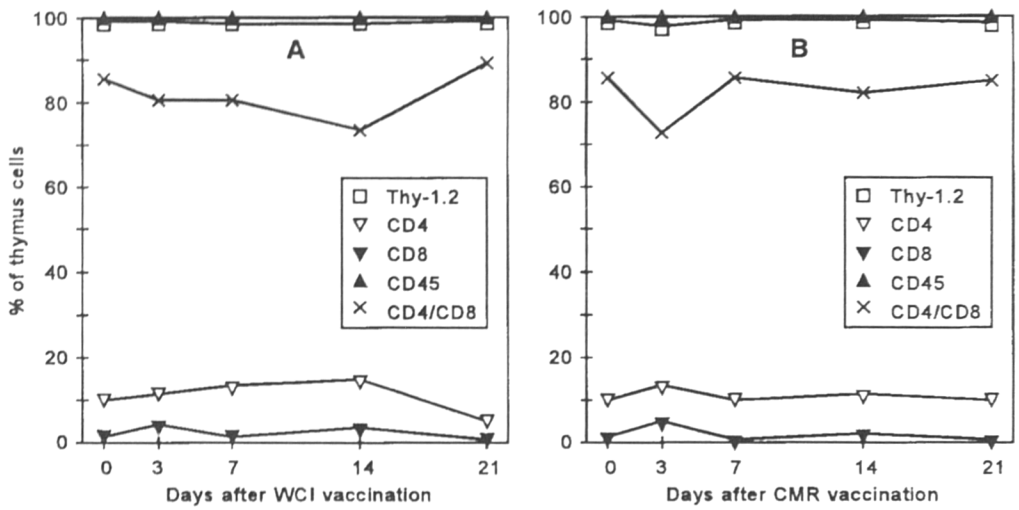

Fig. 4
Percentage of thymus cells from mice immunized with WCI (A) or CMR (B) vaccine expressing various surface epitopes

Table 1. Alterations in the CD4/CD8 cell ratio after injection of mice with *C. burnetii* WCI or CMR vaccine

Days after vaccination[b]	CD4/CD8 cell ratio[a]		
	PBS[c]	CMR	WCI
3	1.28	1.27	1.35
7	1.42	1.58	2.06
14	1.36	1.64	2.04
21	1.65	1.59	2.29

[a]The percentage of CD4 cells divided by the percentage of CD8 cells in the spleens of vaccinated mice.
[b]Spleen cells were collected 3, 7, 14, or 21 days after vaccination.
[c]Mice were injected with PBS containing 100 µg of CMR or WCI.

with WCI or CMR (Fig. 4). In addition, within each of th vaccinated groups, the percentage of thymus cells bearin each marker was relatively constant through the course the experiment.

To determine if *in vitro* hyporesponsiveness of culture lymphocytes was due to an increase in the proportion suppressor T cells relative to helper T cells, the Th/Ts (CD CD8) ratio was calculated (Table 1). At all time points, th CD4/CD8 ratio for spleen cells from WCI-injected mice wa greater than the ratio for control mice. Vaccination wit CMR did not cause the CD4/CD8 ratio to deviate from con trol values.

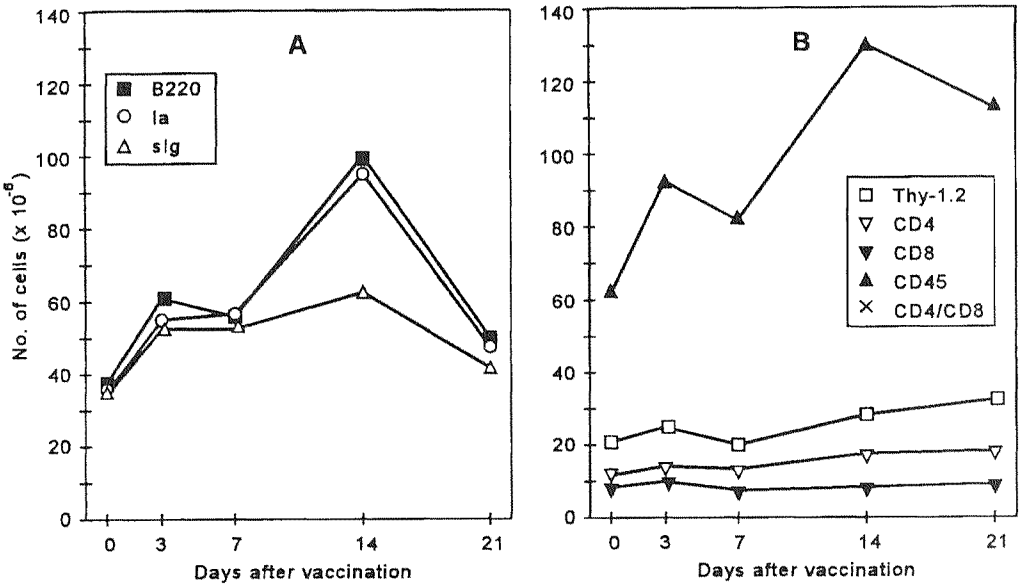

Fig. 5
Number of splenocytes from WCI-immunized mice bearing B cell (A), T cell or leukocyte (B) surface epitopes

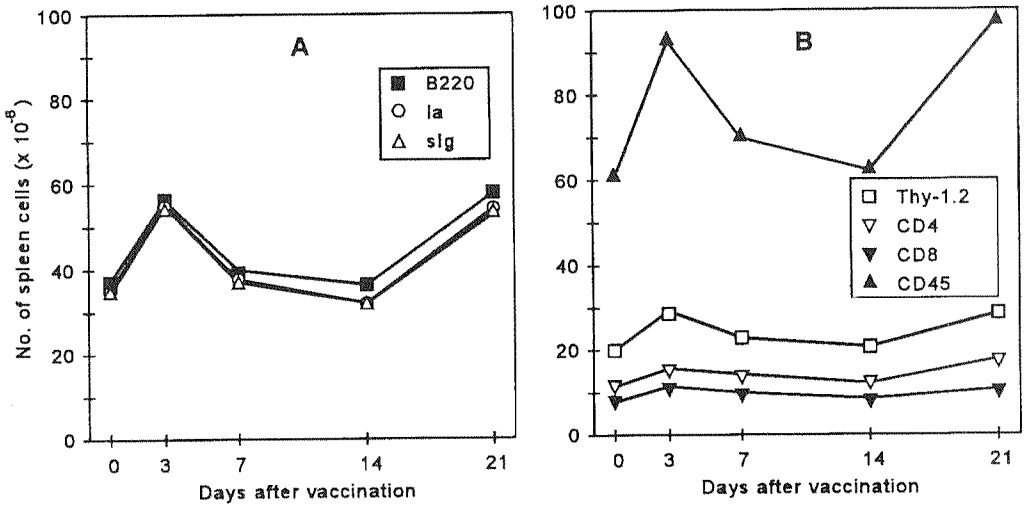

Fig. 6
Number of splenocytes from CMR-immunized mice bearing B cell (A), T cell or leukocyte (B) surface epitopes

Changes in the number of lymphoid cells bearing specific markers

The number of lymphoid cells displaying a particular phenotype was calculated by multiplying the percentage of cells bearing that marker by the average number of nucleated cells per spleen or thymus. Changes in the spleen B cell population were dramatic. Numbers of B-220- and Ia-positive B cells increased from 3.5 x 10^7 to approximately 9.5 x 10^7 cells per spleen 14 days after vaccination with WCI, and decreased to 5.0 x 10^7 cells by day 21 (Fig. 5A). The profiles of cells having B-220 or Ia markers were nearly identical. The numbers of sIg-bearing cells were also similar to the numbers of B-220- and Ia-bearing cells, except on day 14 after vaccination, when only 6.4 x 10^7 sIg-bearing cells were found in the spleen.

The number of spleen cells bearing the CD8 cell surface marker remained constant at approximately 8 x 10^6 cells per

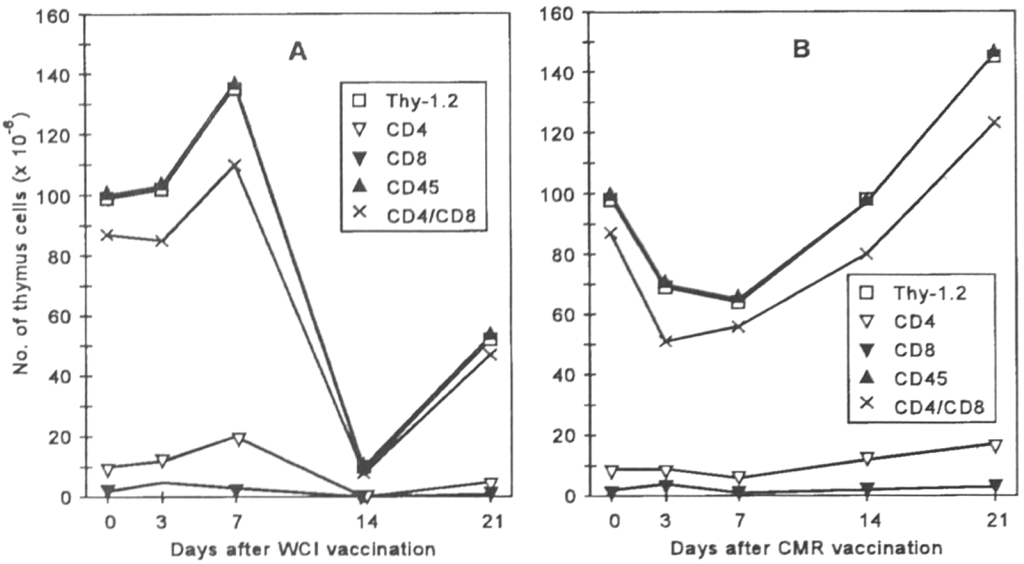

Fig. 7
Number of thymus cells from mice immunized with WCI (A) or CMR (B) vaccine expressing various surface epitopes

spleen in mice vaccinated with WCI (Fig. 5B). However, within 21 days of vaccination, the average number of cells bearing the Thy-1.2 or CD4 markers increased from 2.1 x 10^7 and 1.2 x 10^7 to 3.3 x 10^7 and 1.7 x 10^7 per spleen, respectively. The total number of leukocytes (CD45+) increased from 6.2 x 10^7 to 1.31 x 10^8 cells by day 14 after vaccination.

Changes in the total number of assayed cells were somewhat less dramatic in mice vaccinated with CMR. Cells with B cell markers (B-220, Ia or sIg) exhibited an early (by day 3) and a late rise (day 21) (Fig. 6A). The dynamic changes in numbers of B cells matched changes in the number of splenic leukocytes (CD45+) (Fig. 6B). The total numbers of T cells (Thy-1.2- positive) rose slightly by day 3, followed by a return to preimmunization levels by day 14, before rising again by day 21 (Fig. 6B). This profile was mirrored by cells with CD4 or CD8 phenotypes.

Whereas the percentage of thymocytes from vaccinated mice bearing assayed markers tended to remain relatively constant during the course of the experiment (Fig. 4), total cell numbers of defined populations changed. In WCI-vaccinated mice, numbers of thymocytes bearing the CD45, Thy-1.2, or CD4 and CD8 label increased by day 7 after vaccination, but decreased precipitously by day 14, then increased slightly by day 21 (Fig. 7A). In these mice, the CD4 and CD8 populations exhibited an initial doubling within 7 days of vaccination, then decreased to levels below baseline by day 14. This profile is in marked contrast to that found in CD4 and CD8 populations from CMR-injected mice (Fig. 7B). After an initial decline in the number of CD45- or Thy-1.2-bearing cells until days 3 to 7, there was a substantial increase in the number of cells bearing these markers. The numbers of thymocytes bearing the CD4 marker increased almost two-fold but numbers of cells bearing CD8 remained relatively constant through the course of the experiment.

Discussion

The antibody profiles generated by mice in response to vaccination with WCI or CMR were similar. The anti-phase II antibody response occurred earlier and had greater magnitude than either the anti-phase I or anti-LPS response. This is thought to be due to the absence of antigen masking by phase I LPS on the surface of phase II microorganisms (Hackstadt, 1988) and the greater immunogenicity of the phase II antigenic determinant. Overall, WCI vaccination induced somewhat higher levels of antibodies directed against phase I and phase II antigens. Chloroform-methanol extraction might have removed some of the adjuvant effects found in phase I cellular *C. burnetii*.

Vaccination with WCI decreased the percentage of spleen cells with B cell phenotypes, beginning on day 7. However, there was an increase in the number of cells with B-220 and Ia surface markers between days 7 and 14. B cells exhibited the greatest rate of numerical increase of all cell phenotypes assayed. This correlates with the appearance of significant anti-*C. burnetii* antibody levels. A decline in the

percentage of splenic B cells while that population was increasing numerically indicates that another population of cells was dramatically increasing within 14 days of vaccination with WCI. One population of cells that increased in percentage and in number did not stain with antibody directed against CD45 (a leukocyte marker). We first saw this unusual population of leukocytes on day 7 after vaccination with WCI, and the greatest increase occurred between days 7 and 14 after vaccination. This correlates with maximal *in vitro* immunosuppression. Numbers of CD45-positive cells decreased to only 66% of the spleen cells examined. We are intrigued by our failure to identify 34% of splenocytes that did not express a phenotype found on all leukocytes. Because of hematopoiesis in the mouse spleen, we supposed that these cells might be reticulocytes, but the cells did not stain with antibody directed against reticulocytes (data not shown). Because these cells did not stain with CD45, they were not likely mature leukocytes, but may have been immature cells which did not yet attain cell surface markers characteristic of mature cells. Whether these cells contributed to the *in vivo* pathogenesis or *in vitro* immunosuppression is unknown.

In mice vaccinated with WCI, the number of B cells possessing the B-220 or Ia surface markers was always greater than that of those cells possessing the sIg marker. On day 14 after vaccination with WCI, the lowest percentage of B cells contained the sIg marker. The significance of this is unknown. As B cells differentiate, the antibody is secreted rather than bound to the cell surface, and cells bind less anti-sIg. However, the greatest number of B-220- and Ia-positive B cells and diminished anti-sIg binding in spleens (at day 14) did not correlate with the highest levels of serum antibody measured (day 21). Lack of information on the relative contribution of splenic B cells to levels of serum antibody and the amount of antibody produced per cell during the experiment make any correlation impossible.

In comparison, vaccinating mice with CMR did not decrease the amount of sIg-bearing cells relative to the amount of B-220- and Ia-bearing cells. There were fewer B cells after CMR vaccination than after WCI vaccination. That fewer B cells were secreting similar amounts of *C. burnetii*-specific antibodies *in vivo* after vaccination with WCI suggests that (1) more antibody was secreted per B cell in mice vaccinated with CMR, (2) the antibody response after WCI vaccination was suppressed, or (3) more secreting B cells in mice vaccinated with CMR resided in non-splenic areas than those in mice vaccinated with WCI. Compared to the effects of WCI on splenocytes bearing assayed cell surface markers, vaccination with CMR produced fewer dramatic changes in phenotypic markers. All cells assayed were leukocytes. The profile of the number of leukocytes (CD45-positive) present per spleen of each assay day was mirrored by the profiles of B cells (positive for B-220, Ia, and sIg) and T cells (Thy-1-positive). This indicates that CMR immunization changed the numbers of splenic B and T cells equivalently.

Most important, WCI vaccination did not increase the numbers of T-suppressor cells (CD8) in the spleen or decrease the relative percentage of helper to suppressor cells. Therefore, any involvement of suppressor cells in regulating the immune response after WCI vaccination is likely to be due to activation of resident suppressor cells rather than proliferation of or influx of CD8 suppressor cells to the spleen. Alternatively, excessive levels of cytokines (tumor necrosis factor, interleukin 10, etc.) may have caused the suppressive effects. We do not know if the unidentified spleen cells found 14 days after WCI vaccination participated in immunosuppression.

Although the percentage of thymic leukocytes bearing the various markers remained virtually identical through the course of the experiment in mice vaccinated with WCI or CMR, there were dramatic differences in numbers of thymic leukocytes at specific times after vaccination. In CMR-injected mice, after an initial drop in the numbers of CD45, Thy-1.2, and CD4/CD8-bearing cells, their numbers steadily increased through day 21 after vaccination. However, numbers of "mature" cells bearing the CD4 or CD8 cell surface phenotypes failed to increase in the spleen or thymus. Perhaps sampling later time points would have shown increasing numbers of mature cells. In contrast, there was a precipitous drop in the numbers of thymic leukocytes bearing T cell markers (Thy-1.2) between days 7 and 14 after vaccination with WCI. This coincided with a modest increase in the number of Thy-1 bearing splenocytes beginning 7 days after vaccination. Perhaps splenocytes from WCI-injected mice had shorter life spans than cells from CMR-injected mice and had to replace their numbers from thymic stores at a greater rate. Whether vaccination with WCI caused thymocytes to migrate from the thymus, hindered thymocyte proliferation, or increased the rate of leukocyte death in the thymus is unknown.

In summary, injecting mice with WCI induced greater changes in the number and percentage of spleen and thymus cell leukocyte surface markers when compared to those generated by CMR vaccination or PBS injection. Therefore, CMR may be a safer alternative to WCI vaccine.

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

The views, opinions and/or findings contained in this paper are those of the authors and should not be construed as an official United States Department of the Army position, policy or decision unless designated by other documentation.

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