Establishment of Neospora caninum antigen-specific T cell lines of primarily CD4+ T cells

W. Tuo, W. C. Davis, R. Fetterer, M. Jenkins, P. C. Boyd, L. C. Gasbarre & J. P. Dubey

Animal Parasitic Diseases Laboratory, ANRI, USDA/ARS, Beltsville, MD 20705, USA; Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, WA 99164, USA; Bovine Functional Genomics Laboratory, USDA/ARS, Beltsville, MD 20705, USA

Neosporosis is an important cause of pregnancy loss in cattle worldwide. Protective immunity against Neospora caninum infection may include both cell-mediated (CMI) and humoral immune responses. This study was to establish short-term antigen-specific T cell lines composed of primarily CD4+ T cells from peripheral blood lymphocytes (PBL) of infected cows, which may be used to identify immunodominant antigens for the development of N. caninum vaccines. Crude N. caninum tachyzoite antigen was prepared from in vitro derived N. caninum tachyzoites. Multiple T cell lines were established and maintained for 11 weeks by weekly re-stimulation with N. caninum antigen and antigen-presenting cells. All cell lines responded highly to antigen between weeks 3 and 11. Phenotypically, these cells were composed primarily of CD4+ T cells between weeks 2–8, with a gradual expansion of gamma/delta T cells thereafter. The results indicate that N. caninum-specific T cell lines can be established and maintained without exogenous T cell growth factors and may be used to identify N. caninum antigens. This research will enhance our understanding of bovine CMI to neosporosis and may facilitate development of a proven neosporosis vaccine.

Neospora caninum is an intracellular parasite that causes neosporosis in a number of animal species including cattle (1,2). In the past decade, neosporosis has been recognized as an important cause of pregnancy loss in cattle worldwide. It was estimated that approximately 20% of all bovine abortions were due to neosporosis. Neospora caninum-associated abortion costs the cattle industry in excess of $35 million a year in the state of California alone (3). Although antibody responses have been well documented in N. caninum-infected cows, N. caninum-stimulated cell-mediated immunity is poorly understood. Previous exposure to, or infection by, the parasite does not necessarily provide protection against neosporosis-associated pregnancy loss, in spite of the presence of N. caninum-specific antibody in the circulation. Furthermore, it appears that seropositive cows are more likely to suffer from pregnancy loss than those that are seronegative (4–7). It was reported in different species that N. caninum infection could elicit a measurable cell-mediated immune response with high levels of interferon-gamma (IFN-γ) production (8–10). It appeared that CD4+ T cells (8–10) and type 1 cytokines such as IFN-γ production are critical for the control of neosporosis (11–15). Since N. caninum is an intracellular parasite that requires an antigen-specific cell-mediated immune response or type 1 immune response for protection (16), the objective of the present study was to establish short-term T helper cell lines for subsequent use as antigen-specific T cell lines to screen for immunodominant N. caninum antigens that may be used as vaccine candidates against neosporosis.

Three Holstein dairy cows were immunized subcutaneously twice with killed, whole N. caninum tachyzoite lysate (400 µg/cow/injection) in ImmunoMax SR (Zonagen, Woodlands, TX),
and challenged intravenously with culture-derived live *N. caninum* tachyzoites (NC1 strain) (17). The cows were bled from the jugular vein weekly using vacutainers containing EDTA as anticoagulant. The cows were maintained at the USDA Dairy facility at Beltsville, MD. Animal use and care was approved by the USDA/ARS BARC Animal Use and Care Committee. *Neospora caninum* (NC1) tachyzoite antigen was prepared from cultured *N. caninum* tachyzoites. Briefly, *N. caninum* tachyzoites cultured in bovine macrophages and monkey kidney cell lines were collected, passed through a 27-gauge needle, and filtered. *Neospora caninum* lysate antigen was prepared by five freeze–thawing cycles or sonication with five 10-s pulses (Virsonic Cell Disrupter, The Virtis Company, Gardiner, NY) and protein concentration determined by a BCA protein assay (Bio-Rad). T cell lines were established from PBL of immune cows by weekly re-stimulation with *N. caninum* antigen as described previously (18). T cell line was defined as a short-term cell line that was composed primarily of the CD4+ T cell phenotype. The PBL from peripheral blood of infected cows were prepared by gradient centrifugation using Histopaque (Sigma), washed with Hank's balanced salt solution (Sigma), and then resuspended in complete medium (RPMI 1640 supplemented with 10% foetal bovine serum, 5 µg/mL gentamycin, 5 × 10⁻⁵ M 2-mercaptoethanol). All cell lines were maintained by stimulation with *N. caninum* tachyzoite crude antigen of the NC1 strain and APC. Briefly, PBL (3 × 10⁶ cells per well) were cultured for 7 days in 24-well plates (Fisher Scientific) in 1·5 mL complete medium in the presence of 1000 ng/mL soluble *N. caninum* tachyzoite antigen (18,19). After the first week in culture and weekly thereafter, all cell lines were sub-cultured at 5 × 10⁶ cells/well with 2 × 10⁶ irradiated (3000 rad) autologous PBL as a source of APC. T cell lines were maintained until a significant number of gamma/delta T cell receptor+ T cells emerged. T cells were analysed for phenotype by flow cytometry and tested for antigen-dependent proliferation 7 days following the last antigen stimulation.

The phenotypes of fresh PBL and short-term T cell lines were analysed weekly for surface expression of CD2, CD3, CD4, CD8, and the delta chain of the gamma/delta T cell receptor, using specific monoclonal antibodies (Washington State University Monoclonal Center, Pullman, WA) by flow cytometry as described elsewhere (18). The proliferation assay was performed using round-bottom 96-well plates (Fisher Scientific) for 5 days with PBL, or for 3 days with T cell lines, as described (18). Data are expressed as mean stimulation index (SI), which was calculated by dividing the experiment cpm by the cpm of the medium alone. Overall, the medium alone cpm was 1281 ± 232 for T cell assay and 789 ± 238 for PBL assay. Levels of IFN-γ in cell supernatants were determined by a bovine-specific ELISA (BOVIGAM™, Biocor Animal Health, Omaha, NE).

The PBL from three out of 13 cows proliferated greatly and produced high levels of IFN-γ in response to *N. caninum* antigen stimulation; these cows were chosen as blood donors for the establishment of T cell lines (data not shown). More than 10 short-term T cell lines from each of these three cows were established and maintained for up to 11 weeks by weekly re-stimulation with crude *N. caninum* tachyzoite antigen in the presence of APC. These cell lines proliferated greatly in response to *N. caninum* tachyzoite antigen at concentrations of 100 and 1000 ng/mL, but not to similarly prepared host cell proteins from bovine macrophage M617 (Table 1) or monkey kidney cell CV1 (data not shown). Weekly proliferation assays showed that all cell lines proliferated at low levels in response to *N. caninum* antigen in the first 2 weeks, but responded consistently at high levels between weeks 3 and 11 (Table 1). No significant decrease in proliferation was seen at weeks 9 through 11 when significant levels of gamma/delta+ T cells were present.

| Table 1 Weekly antigen-specific proliferation (expressed as stimulation index) of T cell lines in response to *N. caninum* tachyzoite antigena |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Stimulation                 | 1               | 2               | 3               | 4               | 5               | 6               | 7               | 8               | 9               | 10              | 11              |
| Con A (2·5 µg/mL)           | 42 ± 9          | 57 ± 2          | 57 ± 2          | 31 ± 7          | 27 ± 4          | 42 ± 1          | 40 ± 4          | 34 ± 6          | 69 ± 3          | 47 ± 6          | 15 ± 3          |
| Ag (1000 ng/mL)b            | 13 ± 1          | 35 ± 3          | 35 ± 14         | 53 ± 2          | 47 ± 4          | 47 ± 3          | 38 ± 3          | 40 ± 5          | 66 ± 3          | 53 ± 7          | 28 ± 8          |
| Ag (100 ng/mL)c             | 2 ± 1           | 15 ± 7          | 31 ± 15         | 36 ± 4          | 20 ± 2          | 32 ± 4          | 27 ± 4          | 35 ± 7          | 52 ± 4          | 38 ± 10         | 33 ± 8          |
| Host (1000 ng/mL)c          | 0d             | 1 ± 1           | 1 ± 1           | 0               | 2 ± 1           | 1 ± 1           | 1 ± 1           | 1 ± 1           | 3 ± 1           | 0              |
| APC + Con A                 | 0              | 0               | 0               | 0               | 0               | 0               | 0               | 0               | 0               | 0              |

a Results of three *Neospora caninum* tachyzoite antigen-specific CD4+ T cell lines from each of the three cows are presented. Results are expressed as mean stimulation index ± SEM.

bAg. *Neospora caninum* tachyzoite antigen at a final concentration of 1000 ng/mL.

cHost, host cell (bovine macrophage cell line M617) extract at a final concentration of 1000 ng/mL.

d0, SI less than 1 is considered zero.

eAPC, antigen-presenting cells.
Neospora antigen-driven T cell lines

Table 1; Figure 1). All T cell lines were analysed weekly by single colour flow cytometry using the bovine-specific antibodies against CD2, CD3, CD4, CD8, and the delta chain of the gamma/delta TCR (18). The results showed that total T cells of the bovine PBL were composed of CD4+ T cells (18%) and CD8+ (9%) and gamma/delta+ T cells (12%) (PBL, Figure 1a). However, the percentage of CD4+ T cells in T cell lines increased in response to N. caninum antigen stimulation (week 4, Figure 1a) and was maintained at high levels until after week 8, when gamma/delta+ T cells appeared to gradually outgrow the CD4+ T cells (week 10, Figure 1a). Collectively, the percentages of CD4+ T cells among total cells were low in PBL, but highly enriched after 2–3 weeks of antigen stimulation and remained high thereafter in culture (Figure 1b). Starting from week 9, the percentage of CD4+ T cells in all T cell lines gradually decreased as a result of the increasing percentage of gamma/delta+ T cells (Figure 1a,b), which was consistent with previous reports that gamma/delta+ T cells outgrew CD4+ T cells (18,20).

Neosporosis has been considered an important cause for bovine pregnancy loss in the past decade (1,2). However, no effective vaccine against neosporosis has been developed. Previous reports indicate that cows with high levels of circulating anti-N. caninum antibodies remain susceptible to neosporosis-associated pregnancy loss, suggesting that previous exposure to, or infection with, N. caninum may not necessarily provide protection against the infection. Since N. caninum is an intracellular parasite, host protective immunity may require both cell-mediated and humoral immune responses. Indeed, critical involvement of CD4+ T cells and the inflammatory cytokine IFN-γ in neosporosis has been described in cattle and mice (8–11,13,15), suggesting an important role of cell-mediated immunity to neosporosis. Consistent with previous reports, results in the present study showed that infected cows developed a detectable N. caninum-specific cell-mediated immune response in the form of CD4+ T cell proliferation and IFN-γ production.

To our knowledge, N. caninum antigen-driven T cell lines composed of primarily CD4+ T cells have not been well characterized. One report described the use of such T cell lines to screen for N. caninum tachyzoite antigens, but those T cell lines used in the report (8) were maintained and
expanded with a T cell growth factor, IL-2. Usually, the need for exogenous T cell growth factors to maintain the survival and/or expansion of T cell lines may reflect reduced dependence of the T cells on specific antigens. In contrast, our T cell lines were maintained with N. caninum tachyzoite antigen only in the presence of antigen-presenting cells. They were highly N. caninum tachyzoite antigen-dependent, as shown by consistent low level proliferation in medium alone or in host cell extract controls. These T cell lines were maintained in culture for up to 11 weeks without significant decrease in levels of proliferation due to variations in cell composition. It was clearly shown that PBL had relatively lower levels of antigen-specific proliferation in comparison with T cell lines weeks 2 through 11. It is true that PBL contained low levels of CD4+ T cells (18%) and the N. caninum antigen-specific CD4+ T cells may be only a small percentage of the total CD4+ cell pool. However, antigen-specific CD4+ T cells were highly enriched in T cell lines by selective antigen stimulation and antigen non-specific T cells may have died out due to the lack of stimulus. Therefore, even for week 11 T cell lines, N. caninum antigen-specific CD4+ T cells remained high (~30%) and thus, the actual number of N. caninum antigen-specific CD4+ T cells may be enough to support antigen-specific proliferation to the levels shown.

The purpose of this study was to develop N. caninum antigen-specific T cell lines that can be used to identify immunodominant antigens for vaccine development. Thus, T cell lines at weeks 3 through 8 should be used, since T cell lines during this period contained the most CD4+ cells. The immunodominant antigens identified by N. caninum antigen-specific T cell lines may effectively elicit both cell-mediated and humoral immune responses against N. caninum infection. The results of this research will enhance our understanding of bovine cell-mediated immunity to and may facilitate the development of vaccines against bovine neosporosis.

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