ABSTRACT  
We have utilized a newly developed culture system to study the properties of antitumor CD4+ T-cells relevant to the rejection of syngeneic methylcholanthrene sarcomas. Fresh syngeneic dendritic cells prepared from spleen, then pulsed with crude lysates of methylcholanthrene sarcomas, evoke antigen-specific proliferation by CD4+ but not by CD8+ T-cells from tumor-immune mice. Unfractionated splenocytes display similar antigen presenting capacity if they are not irradiated before the pulse with tumor lystate. CD4+ T-cells from mice immunized to individual methylcholanthrene sarcomas proliferate cross-reactively to dendritic cells pulsed with fresh tumor digests, but not to dendritic cells pulsed with cultured tumor cells. This apparent shared recognition of sarcoma lysates was demonstrated to be a result of sensitization to bacterial collagenase digestion during the immunization procedure. Therefore, the murine CD4+ T-cell response to tumor immunization is similar to the CD8+ response in that sensitization occurs predominantly to tumor specific transplantation antigens rather than to shared tumor antigens. Strategies to avoid artifactual tumor cross-recognition by CD4+ T-cells are discussed.

INTRODUCTION  
It has long been recognized that mice vaccinated to weakly immunogenic MC2 sarcomas can develop specific resistance to subsequent challenge with the same sarcoma (1). This immune protection requires both CD4+ and CD8+ T effector cells (2). Development of cross-reactivity to other syngeneic sarcomas is seldom observed or is incomplete, indicating that TSTA rather than tumor-associated antigens are primarily responsible for MC sarcoma rejection following immunization (3). CD8+ T-cells derived from mice immunized to syngeneic MC sarcomas often fail to show cross-recognition of other sarcomas in 3Cr-release lytic assays or cytokine-release assays, and do not show cross-protection in adoptive therapy experiments (4). Similarly, CD8+ T-cells grown from tumor digests (tumor infiltrating lymphocytes) often manifest a similar absence of cross-recognition (5). In contrast, CD8+ T-cells cultured from the lymph nodes of mice bearing progressive syngeneic MC sarcomas often show cross-recognition of other sarcomas in vitro, as well as cross-protection in adoptive therapy (4, 6). Technical problems previously limited the study of tumor-immune CD4+ T-cells in vitro. In the present report, we describe a newly developed culture technique to demonstrate that, similarly to CD8+ T-cells, CD4+ T-cells derived from tumor-immune mice predominantly recognize unshared tumor antigens. Apparent cross-reactive antigens on tumors are shown to be artifacts of tumor digestion in bacterial collagenase, giving rise during tumor vaccination to collagenase sensitization.

MATERIALS AND METHODS  

Mice. Female C57BL/6 and BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) and National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). Animals were used between ages 8 and 12 weeks.

Murine Tumors. All tumors were of C57BL/6 origin; the MC sarcomas 203 and 207 and adenocarcinoma MC38 were maintained in vivo by serial s.c. transplantation; the sarcomas were serially passed 9 times before reinitiating with early passage cryopreserved tumor mice. WP4 is a cultured clone from the MC sarcoma 205, subsequently passed in mice. The Lewis lung carcinoma was obtained from the Frederick Cell Repository. All tumors were demonstrated free of viral pathogens and Mycoplasma by the National Cancer Institute-Frederick Cancer Research and Development Center.

Culture Medium. All assays and cultures were performed in CM consisting of RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum (Biofluids), 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin sulfate, 2 µg/ml fungizone, 0.05 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1 µg/ml indomethacin.

Tumor Digestion and Cell Line Derivation. Serially passaged s.c. tumors were aseptically harvested and finely minced, then enzymatically digested in 50 ml of Hanks' balanced salt solution containing DNase type IV (0.1 mg/ml), collagenase type IV (1 mg/ml), and hyaluronidase type V (2.5 units/ml) (Sigma, St. Louis, MO), with constant stirring over 2 h at 25°C. After nylon mesh filtering and 2 washes in Hanks' balanced salt solution, the resulting single cell suspensions were used either for immunizations or as a source of tumor antigen.

Murine Tumor Immunizations. Syngeneic mice were immunized to weakly immunogenic MC sarcomas as described previously (1, 2). Naive mice were first inoculated intradermally with 106 viable syngeneic sarcoma cells mixed with 100 µg formalin-killed Corynebacterium parvum; all mice developed small tumors which rapidly regressed. Three weeks following the initial inoculum, all mice received intradermal challenges with 2 x 106 viable tumor cells (without C. parvum); 10–60% of mice rejected these challenges dependent on a particular sarcoma’s immunogenicity. Three weeks following this challenge, tumor-free mice received 106 viable tumor cells intradermally; mice which rejected these challenges (generally 100%) were designated hyperimmune, and used 3 weeks after the last tumor challenge as a source of immune spleens.

Tumor Antigen. Freshly digested tumor cell suspensions were either used directly as a source of antigen or first cultured 1 to 4 weeks in CM without indomethacin. To prepare tumor antigen, freshly digested or cultured tumor cells were snap-freeze-thawed (in CM without dimethyl sulfoxide) twice in rapid succession, then refrozen and stored in liquid nitrogen for later use. No viable tumor cells were detectable in these FT tumor lysates after the final thaw.

Dendritic Cell Preparation. Splenic DC were freshly prepared from naive C57BL/6 mice following the method of Girolomoni et al. (7). After partial purification with DNase treatment and Percoll gradient fractionation, DC were resuspended in CM and placed in 25-cm² Falcon 3013 flasks, 3 spleen equivalents per flask. Nonadherent cells were discarded 2 h later, leaving partially purified adherent DC in culture. FT syngeneic tumor cell lysates were immediately added (2 x 106 tumor cell equivalents/flask), and antigen pulsing allowed to proceed for 18 h. DC were then harvested, having detached from the flasks during overnight incubation. Each group was counted, irradiated (1000 R) (8), then cocultured with CD4+ T-cells in proliferation assays.

T Cell Subset Preparation. While DC were in preparation, fresh CD4+ T-cells were prepared from the spleens of naive or tumor-immune mice.
Splenocytes were first depleted of red blood cells with ammonium chloride-potassium lysing buffer. Negative selection immunoaffinity columns (Pierce Chemical Co., Rockford, IL) were then utilized with a single modification: the provided anti-CD8 reagent or purified anti-CD4 (GK1.5; American Type Culture Collection, Rockville, MD) was diluted in CM conditioned by M5/114 hybridoma (anti-I-A\(^{b}\); American Type Culture Collection) to facilitate quantitative depletion of \(I^a\) cells (9).

**Proliferation Assays.** Flat-bottomed 96-well microtiter plates (Costar) were utilized. Each well received \(1 \times 10^5\) \(CD^4^+\) T-cells. DC were added to achieve a final CD4:DC ratio of 20:1 or 40:1. Each well contained a final volume of 0.2 ml CM. Control groups routinely performed included: CD4\(^+\) T-cells cultured without DC; pulsed irradiated DC cultured without CD4\(^+\) T-cells; each CD4\(^+\) T-cell group with 3 µg/ml PHA (Sigma); each CD4\(^+\) T-cell group sham-pulsed (i.e., cultured in CM only); each DC group with fresh BALB/c (allogenic) CD4\(^+\) T-cells; each DC group with fresh naive C57BL/6 (syngeneic) CD4\(^+\) T-cells. Cultures were continued for a total of 5 days, except for PHA control groups, which received only 3 days' culture. At 18 h prior to assay completion, tritiated deoxythymidine (1 µCi/well) (Amersham, Arlington Heights, IL) was added to each microtiter well. At completion, plates were harvested with a semiautomated LKB cell harvester, and measured by a LKB/Wallac 1205 Betaplate liquid scintillation counter (Gaithersburg, MD). Responses were reported as mean cpm ± SEM from triplicate samples, and analyzed by Wilcoxon rank sum analysis.

**Fluorescence-activated Cell Sorter Analysis.** Cells were routinely analyzed on a Becton-Dickinson FACScan or FACStar Plus using conjugated direct antibodies and subclass matched controls obtained from Becton-Dickinson (Lincoln Park, NJ) and Pharmingen (San Diego, CA). Cells were pre-treated with unconjugated anti-CD32 (2.4G2) to block FcR binding by conjugated antibodies (Pharmingen).

**RESULTS**

**Control Group Analysis of 2-Step CD4\(^+\) T-Cell Proliferation Assay.** A representative experiment illustrating all routine control groups is shown in Fig. 1. Irradiated DC alone showed negligible proliferation at Day 4 in culture, whether or not pulsed with FT-2C03 tumor antigen. CD4\(^+\) T-cells alone also showed negligible proliferation. When naive BALB/c CD4\(^+\) T-cells were cocultured with each DC group, strong proliferation was seen; this confirmed that each DC group manifests both class II expression and costimulatory function. Each CD4\(^+\) T-cell group was also cultured with sham-pulsed DC and PHA to confirm proliferation potential. Naive CD4\(^+\) T-cells showed consistently low level proliferation when cocultured with each irradiated DC group. Finally, CD4\(^+\) T-cells from mice immune to the MC-203 tumor showed low level proliferation to sham-pulsed irradiated DC. Taken together, these control groups enabled the demonstration of significant antigen-specific proliferative effects when MC-203 immune CD4\(^+\) T-cells were cocultured with MC-203-pulsed, irradiated DC (Fig. 1, Group \(L\) versus Group \(M\), \(p = 0.0248\)).

**Tumor-immune CD4\(^+\) T-Cells but Not CD8\(^+\) T-Cells Show Specific Proliferation to Tumor-pulsed DC.** When CD4\(^+\) and CD8\(^+\) T-cell subgroups were each prepared from both naive and tumor-immune mice, only tumor-immune CD4\(^+\) T-cells demonstrated specific proliferation when cocultured with tumor-pulsed DC compared to sham-pulsed DC (Fig. 2, Group \(G\) versus Group \(H\), \(p = 0.0248\)). This proliferation can be blocked by adding anti-Class II or anti-CD4 antibody 12 h after initial coculture (data not shown). Mixing experiments which compared adding either naive or tumor-immune CD8\(^+\) T-cells to tumor-immune CD4\(^+\) T-cells demonstrated nonspecific augmentation of proliferation (Fig. 1, Group \(J\) versus Group \(L\), \(p = 0.4136\)). In other experiments not shown, irradiation of the tumor-immune CD4\(^+\) T-cell subset completely abrogated the proliferative response of either added CD8\(^+\) T-cell group (9).

**Nonirradiated but not Preirradiated Splenocytes Contain the Antigen-processing Capacity of Partially Purified DC.** Several previously published efforts to expand antitumor CD4\(^+\) T-cells in vitro had used preirradiated (3000 R) unfractionated splenocytes as a source of APC for antitumor CD4\(^+\) T-cells (10, 11). Even though a dendritic cell subpopulation is present in such unfractionated splenocytes, such APC have not proved to be an effective way to present tumor lysates to freshly harvested immune CD4\(^+\) T-cells (10). We compared the antigen-presenting function of such previously preirradiated (3000 R) unfractionated splenocytes to our standard preparation of tumor-pulsed splenic DC in which DC are irradiated only after the lysate-pulse period. In addition, in order to assess the effect of preirradiation on naive unfractionated splenocytes, the latter were either preirradiated with 3000 R, then pulsed for 8 h with FT tumor lysate, or first pulsed with tumor lysate, then irradiated. Each APC group was then cocultured with CD4\(^+\) T-cells. As shown in Fig. 3, unfractionated splenocytes did have the capacity to process and present tumor lysate, but this proved to be a radiosensitive event.
CD4⁺ T-Cells from Mice Immunized to Individual MC Sarcomas Proliferate Cross-Reactively to DC Pulsed with Fresh Tumor Digests, but not to DC Pulsed with Cultured Tumor Cells. As shown in Fig. 4, CD4⁺ T-cells obtained from mice immunized to MC-203 or MC-207 showed low level proliferation to sham-pulsed DC, but strong proliferation to DC pulsed with any syngeneic tumor tested, including nonsarcomas, when fresh tumor digest was the source of tumor antigen. In contrast, when tumor digestes were cultured in vitro for 1 to 3 weeks prior to use as a source of FT antigen, no cross-reactive proliferation was seen. In multiple experiments not shown, CD4⁺ T-cells from MC-203 immune mice consistently failed to proliferate cross-reactively to DC pulsed with other cultured syngeneic MC sarcomas, namely MC-207, MC-105, MC-205, WP4, and MC-102. We observed similarly selective proliferation by CD4⁺ T-cells from mice immune to MC-207, MC-105, and WP4 when cultured tumor cells were used as stimulators in vitro.

Cross-Reactive Tumor-immune CD4⁺ T-Cell Proliferation Is Caused by Immunization to Collagenase. DC were pulsed for 18 h with either collagenase, hyaluronidase, or DNase, or pulsed with FT fresh tumor digestes or cultured tumor cells, then cocultured with CD4⁺ T-cells either from syngeneic mice immune to MC-203 tumor, or from naive alllogeneic (BALB/c) mice. As seen in Fig. 5, DC pulsed with collagenase, but not hyaluronidase or DNase, evoked strong proliferation from MC-203 immune CD4⁺ T-cells, as did all DC groups pulsed with fresh collagenase-digested tumor preparations. DC pulsed with cultured tumor cells did not evoke strong proliferation except for the relevant tumor cell line, MC-203. Importantly, all DC groups evoked strong proliferative responses by naive BALB/c CD4⁺ T-cells, indicating adequate antigen-presenting function in all DC preparations. Additionally, all DC groups evoked negligible proliferative responses from naive C57BL/6 CD4⁺ T-cells (latter data not shown).

DISCUSSION

The absence of cross-protection to other syngeneic sarcomas after mice are immunized to particular sarcomas has long suggested that recognition of TSTA often plays the central role in the rejection of these tumors. Such rejections are dependent upon both CD4⁺ and CD8⁺ T-effector cells (2). Previous work had demonstrated that antitumor CD8⁺ T-cells taken from tumor-immune mice or grown from tumor digestes (tumor-infiltrating lymphocytes) often fail to recognize shared sarcoma antigens in vitro and fail to show cross-reactive therapeutic effects when used in adoptive transfer experiments (1, 3–5). Due to technical obstacles, it was previously difficult to characterize the CD4⁺ T-cells involved in MC sarcoma rejections. The present work demonstrates that CD4⁺ T-cells taken from tumor-immune mice also predominantly recognized unshared tumor antigens. It remains to be determined whether both CD4⁺ and CD8⁺ T-cells recognize epi-
topes derived from a single TSTA in the case of each MC sarcoma. It also remains to be determined whether CD4+ T-cells from tumor-bearing mice recognize shared tumor antigens in the pattern observed for CD8+ T-cells from tumor-bearing lymph nodes (4, 6).

The results presented here underscore the danger that seemingly shared tumor antigens may often represent experimental artifact. Mice inoculated with tumor cells freshly digested in clostridial collagenase develop a prominent immune response to that enzyme. No such response has been observed for other digestive enzymes, which are bovine rather than bacterial in origin. Therefore, DC preparation methods which begin with collagenase digestions are likely to generate profound artifacts in this type of tumor model. The repeated washing of tumor cells following digestion does not fully remove the collagenase antigenic activity, but culturing digested tumor cells for several days eliminates the collagenase activity, as demonstrated in Fig. 5 by the loss of cross-reactive tumor recognition. In other experiments not shown, we have demonstrated that tumor-immune CD4+ T-cells can be expanded in culture to enrich for either tumor-recognizing or collagenase-recognizing subpopulations.

Although tumor cells grown chronically in culture do not carry the risk of generating collagenase sensitization when used as a source of tumor for vaccines, animals given repeated injections of cultured tumor cells are apt to develop sensitization to culture medium proteins. In this instance, CD4+ T-cells taken from mice immunized to tumor cultured cell lines show an abnormally high proliferative response to "sham-pulsed" dendritic cells prepared in the same culture medium (e.g., RPMI 1640 with 10% fetal calf serum) used to culture the tumor lines (data not shown). Our current strategy to prepare tumor cells for vaccination involves an initial tumor digestion (of necessity in collagenase) followed by several days' culture in RPMI 1640 supplemented with 0.5 to 1.0% non-heatinactivated syngeneic mouse serum. This culture period facilitates catabolism of collagenase antigen, and adds no new foreign proteins to confound vaccinations.

Either Langerhans cells or spleen DC pulsed with crude FT tumor preparations can be used to present specific tumor antigens to CD4+ T-cells (9). Fresh unfractionated splenocytes also possess significant antigen-presenting capacity, but this is impaired by irradiation prior to the antigen pulse period. Previous investigators studying antigen-specific tumor CD4+ T-cells used unfractionated splenocytes as their source of APC; however, the splenocytes were irradiated prior to antigen pulsing (10, 11). We believe that this partly explains the historical difficulty associated with growing specific antigen CD4+ T-cells in vitro.

In numerous experiments, we have never observed tumor-specific CD8+ T-cell proliferation to be evoked by tumor lysis-pulsed DC; this is true whether or not recombinant interleukin-2 is added to the assay (data not shown). This suggests that DC process and present crude tumor antigen, in vitro at least, solely in a Class II context, and parallels the experience of others who studied DC presentation of exogenous protein antigens such as ovalbumin (12). Since CD4+ T-cells can function effectively in vivo against Class II nonexpressing tumors, including the MC sarcomas described in the present study (2, 11, 13), it is possible that APC within solid tumors process and present exogenous tumor debris in a Class II context to CD4+ T-effector cells, triggering cytokine release which leads to accumulation and activation of CD8+ T-cells (14, 15) as well as accessory cells such as tumoricidal macrophages and lymphokine-activated killer cells (16-18). Such accessory cells kill tumor targets independent of tumor cell Class I and Class II expression (16, 17), and may explain how CD4+ T-cells achieve effectiveness in vivo against Class II nonexpressing tumors (11, 13) as well as Class I nonexpressing tumors (17). We are currently using our culture system to expand specific antigen tumor CD4+ T-cells and investigate their mechanism of tumor rejection in vivo (19).

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CD4+ T-Cells from Mice Immunized to Syngeneic Sarcomas Recognize Distinct, Non-Shared Tumor Antigens

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