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Cytotoxic T lymphocytes raised against a subdominant epitope offered as a synthetic peptide eradicate human papillomavirus type 16-induced tumors

Previously, we have shown that immunization with human papillomavirus (HPV) type 16-derived cytotoxic T lymphocyte (CTL) epitope E7 49–57 (RAHYNIVTF) renders C57BL/6 mice insensitive to tumors formed by HPV16-transformed cells. In this study, we provide evidence that E7 49-57 is expressed as a subdominant CTL epitope on HPV16-transformed C57BL/6 cells. Using acid peptide elution, it is shown that HPV16-transformed cells express another CTL epitope, besides E7 49-57, which appears to be dominant. We demonstrate that a CTL line raised against the subdominant CTL epitope, offered as synthetic peptide E7 49–57, eradicates established HPV16-induced tumors in mice. Our data show that synthetic peptide-induced CTL can be applied successfully *in vivo* against (virus-induced) tumor, and emphasize that subdominant CTL epitopes are useful targets for immunotherapy. Furthermore, it is illustrated for the first time that HPV16-specific CTL interfere directly with HPV16-induced tumors.

1 Introduction

Human papillomavirus (HPV) type 16, in particular the viral oncogene products E6 and E7, are believed to play a causative role in cervical cancer [1]. Development of HPV16-associated (pre)malignant lesions is enhanced in individuals with impaired T cell responses [2–5], suggesting that cellular immunity impedes HPV 16-associated tumor development. CTL are important effectors of protection against (virus-induced) cancer [6, 7]. They lyse target cells upon recognition of specific peptides presented at the cell surface by MHC class 1 molecules [8]. In the case of virus-infected cells, these peptides originate mainly from intracellular viral proteins [9, 10], which are degraded in the cytosol and bound specifically by class I MHC molecules, followed by transport to the cell surface [11].

Previously, we have identified an MHC class I H-2D^b-binding peptide, HPV16 E7 49-57 (RAHYNIVTF), that elicited CTL responses in C57BL/6 (B6) (H-2^b) mice [12]. Vaccination with synthetic peptide E7 49–57, either mixed with IFA [12] or loaded on dendritic cells [32], specifically prevented tumor formation by HPV16-transformed B6 mouse embryo cells (MEC) (C3 cells). Protection was associated with expansion of peptide-specific CTL, which upon stimulation from memory cells, were capable of lysing C3 cells *in vitro* [12], presumably as the result of specific recognition of peptide E7 49–57 presented by class I MHC H-2D^b molecules on the tumor cell membrane. Therefore, CTL raised against synthetic peptide E7 49–57 should be able to recognize naturally processed E7 49–57 peptide expressed on HPV16-transformed C3 cells and cure established C3 tumors when injected in tumor-bearing B6 mice.

In this study, we describe the generation and characterization of a stable CTL line raised against synthetic peptide E7 49–57. This CTL line specifically recognizes E7 49–57 as a naturally processed peptide on HPV16-transformed C3 cells and eradicates established C3 tumors in B6 nude mice. In addition, we show that CTL epitope E7 49–57 is immuno-subdominantly expressed on C3 cells, inasmuch as CTL raised against C3 cells recognize another naturally processed peptide. The data presented in this study are the first examples of CTL-based immunotherapy against established HPV16-induced tumors, supporting the thought that T cells are involved in the control of cervical cancer. Furthermore, our data stress the feasibility of generating tumor-specific CTL against epitopes of choice, in particular subdominant epitopes, offered as synthetic peptides.

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Abbreviations: Ad5: Adenovirus type 5 **B6(MEC):** C57BL/6 (mouse embryo cells) **HPV16:** Human papillomavirus type 16 **i.t.:** Intratumoral

Key words: Cytotoxic T lymphocytes / Peptide / Immunodominance / Human papillomavirus 16 / Adoptive transfer

2 Materials and methods

2.1 Cell lines, generation of CTL lines/clones and culture conditions

Cell line C3 was obtained from HPV16-/EJras-transformed B6MEC [12], and cell line Xhoc3 from adenovirus type 5 (Ad5) E1-transformed B6MEC [13]. Cell line p8 was obtained from HPV16-transformed B6MEC. In contrast to C3 cells, p8 cells do not form tumors in B6 mice. Cell line EL4 is derived from B6 thymoma cells [14]. All cells were cultured in Iscove's modified DMEM supple-

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Table 1. Synthetic peptide E7 49–57-induced CTL lyse E7 49–57 peptide pulsed EL4 cells as well as HPV16-transformed C3 cells, whereas C3 cell-induced CTL lyse only C3 cells

CTL	E: I[d]	Target cells				
		EL4+ Ad5 E1B 192–200	EL4+ HPV16 E7 49–57	a[a]	Xhoc3[b]	p8[c]
FaC3	16	- 1[e]	ffij	87	4	
	S	-ft	98	74	3	n.t.
	4	-3	90	59	0	
	2	-5	88	53	2	
7G1	16	10	2	33	1	66
	H	-8	(i	28	-	55
	4	-A	7	24	-	46
	2	-V	2	25	1	37
01	Eft	84	K	8	49	
	8	59	4	2	44	n.t.
	4	45	2	1	44	
	2	22	3	5	29	

a) O: HPV16/EJras-transformed B6 MEC.
h) Xhoc3: Ad5 E1-transformed B6 MEC.
c) pS: HPV16-transformed B6 MEC.
d) Effector to target ratio.
e) Percentage specific lysis. The experiment shown is a representative of six independent experiments. SD of triplicate wells never exceeded 10 %.of the calculated mean percentage specific lysis. which is depicted.

mented with FCS, antibiotics and 2-ME. as described [12]. CTL line FaO was made by immunizing B6 mice s.c. with 100 μg synthetic E7 49–57 peptide mixed with IFA [12]. After 14 days. T cell-enriched spleen cells [15] were cultured in tIK presence ol E7 49–57 peptide-loaded RMA-S cells (50 μM, 2 h in serum-free medium) [15, 16] tn 96-well plates. 2 x 10^{5} and 5 x 10^{4} cells/well. respectively After 5 days, viable cells were stimulated weekly with IFN-γ-pretreated (U) IU/ml. 48 h) C3 cells. From dav 12 on, the equivalent of 60 IU/ml rIL-2 derived from the supernatant of C"on A-treated rat spleen cells was added to the cultures [17]. which was gradually replaced hy 200 IU/ml rIL-2 from week X onwards. Using limiting dilution CTL clone 7G1 was obtained from B6 mice immunized with 10 IFN-γ-pretreated C3 cells inactivated by irradiation, suspended in 200 μl PBS. and injected s.c. twice with a 2-week interval. CTL clone 01. specifically recognizing peptide Ad5 E1B 192–200. was raised against mutant Ad5 EJ-transformed B6 MEC [18]. CTL clones 7G I and 01 were stimulated weekly with IFN-γ-pretreated C3 and Xho3 cells. respectively. in the presence of 50 IU/ml rIL-2. All CTL were CD4 CD8 . as demonstrated by FACS (data not shown).

2.2 Europium (Eu^{3+})-release cytotoxicity assay

Eu^{3+} labeling and Eu^{3+}-release assay were performed as described [12, 19]. In the case of peptide loading, labeled target cells wen? incubated wilh 0.5 μM peptide prior to addition 10 the effector cells. Alter 4 h at 37°C, supernatants were analyzedin a 1234 Delfia® fluorometer (Wallac, Turku. Finland]. The mean percentage specific Eu^{3+} release of triplicate wells was calculated as follows: % specific lysis - (cpm experimental release • cpm spontaneous release)/(cpm maximum release - cpm spontaneous release) x 100.

2.3 Add peptide elution

Acid peptide elution was performed as described [20, 21], with slight modifications. IFN-γ-pretreated C3 cells (3 x 10^{7}: 10 IU/ml. 48 h) were washed and incubated with 5 ml acid buffer solution (0.131 M citric acid, 0.06! M $Na_2HPO_4 \cdot 2H_2O$, pH 3.3) for 3 min at room temperature. Peptides in the supernatant of 8 x JO" cells were concentrated and desalted on a Sep-pak C18 column (Millipore. Milford, MA). Peptide material was eluted with 1 mi of 60% acetonitrile in H_2O, lyophilized and dissolved in 100 μl H_2O. The reconstituted material was fractionated using an acetonitrile gradient on a C18 reverse-phase HPLC' column (Pharmacia LKB. Roosendaal. The Netherlands). Individual fractions were lyophilized. reconstituted with PBS to 100 [d and analyzed in a Eu^{3+}-release assay. Fur each CTL tested, three wells with 5 μl/well of each fraction were incubated with Eu^{3+}-labeled EL4 cells for 30 min before CTL were added.

2.4 Mice and adoptive transfer

T cell-deficient B6 nude mice (Bolmholtgard. Ry. Denmark). held under specific pathogen-free conditions at TNO-PG (Leiden, The Netherlands), were inoculated s.e. each with 5 x 10" O cells at the right flank. When tumors were about 200 mm^3. therapy was started via adoptive transfer of 25 x 10" CTL injected i.v. in the tail vein or intratumorally (i.t.) directly into the tumor in 300 |d or 50 μl cell suspension, respectively. This was repeated weekly for 3 weeks and combined with s.c. administration of 10[5] IU rIL-2 in IFA in the left flank. THe same amount of IL-2 was also given every 3rd day after CTL injection.

3 Results

3.1 Synthetic peptide HPV16 E7 49–57-induced CTL specifically lyse E7 49-57 peptide-loaded EL4 cells as well as HPV16-transformed C3 cell*

CTL line FaC3, induced against synthetic peptide HPV16 E7 49-57 and stable in culture for more than i year, was

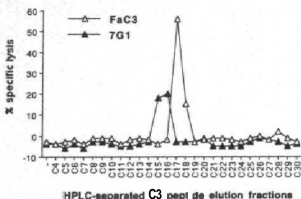

Figure 1. HPV16 E7 49–57 peptide-induced CTL and C3 cell-induced CTL, respectively, recognize different HPLC-separated peptide fractions derived from acid-eluted HPV16-transformed C3 cells. Peptide material from the supernatant of a total of 8 x 10^9 acid (pH 3.3) buffer-treated C3 cells was separated by reverse-phase (C18) HPLC. A proportion of each fraction was incubated with Eu^{3+}-labeled EL4 cells, after which either the E7 49-57 synthetic peptide-induced CTL line FaC3 or C3 cell-induced CTL clone 7G1 was added. A standard Eu^{3+}-release assay was carried out, followed by calculation of the percent specific lysis. A representative experiment out of three is shown.

tested for target cell recognition. In a representative experiment, FaC3 specifically lysed syngeneic EL4 cells pulsed with **E7** 49-57 peptide, as well as C3 cells that express the HPV16 E7 gene [12] (Table 1). EL4 cells loaded with the D^b-restricted CTL epitope Ad5 E1B 192–200 as well as Ad5 El-transformed B6MEC (Xhoc3 cells) were not lysed by FaC3. whereas they were by Ad5 El B 192–200-specific CTL clone 01 [18] (Table i). In titration experiments, the affinity of FaC3 for its cognate peptide was lost between JO and I pM (data not shown), which is comparable with other known murine CTL clones [18, 22].

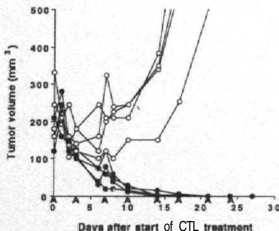

Figure 2. Intratumoral administration of HPVI6 E7 49-57-specific CTL line FaC3 eradicates tumors induced hy HPV 16-transformed C3 cells in T cell-deficient B6 nude mice. The results shown are from a representative experiment of two. in which B6 nude mice were inoculated s.c. with 5 x 10^6 HPV16-transformed C3 cells. When tumors of about 200 mm^3 were formed, mice were injected i.t. weekly for 3 weeks (closed arrows) with 25 x 10^6 HPV16 E7 49–57-specific CTL line FaC3 (closed circles, n = 4) or 25 x 10^6 Ad5 E1B 192–200-specific CTL clone 01 (open circles, n = 4). At the same time, the mice received 10^5 IU IL-2 s.c., which was repeated 3 days after each CTL injection (open arrows). The tumor volumes are shown from the first day of CTL treatment onwards.

3.2 HPV16-transformed C3 cell-induced CTL specifically lyse HPV16-transformed C3 cells but do not lyse E7 49–57 peptide-loaded EL4 cells

In the same experiment, CTL obtained from HPVI6-transformed C3 cell-immunized mice were tested. CTL clone 7G1. a representative of many CTL bulks and clones obtained from several independent C3 cell immunizations over the years (one of which was described before [23]), specifically lysed C3 cells. Surprisingly, E7 49–57-pulsed EL4 cells were no! lysed by 7G1 (Table 1), suggesting that HPV16-transformed C3 cells express at least two **CTL** epitopes. Of these two epitopes, E7 49–57 is subdominant in the sense that upon C3 cell immunization (in contrast to E7 49-57 peptide vaccination), no CTL were detected that recognize this particular peptide. The HPV 16-transformed B6 MEC line p8, which does not contain EJras, was also lysed by 7GI, implying that the immunodominant epitope expressed on C3 cells is HPV16 specific (Table I). Similar target cell recognition patterns were observed when specific TNF-α release by the CTL tested was monitored (data not shown).

3.3 Different peptide fractions obtained from HPV16-transformed C3 cells are specifically recognized by E7 49–57 peptide-induced CTL and C3 cell-induced CTL, respectively

To show that HPV16-transformed C3 cells express at least two different CTL epitopes, among which is E7 49–57, we subjected 8 x 10^9 cultured C3 cells to mild acid treatment. As a result, peptides are eluted from cell surface MHC class I molecules [21]. This preparation was fractionated by reverse-phase HPLC. Individual fractions were analyzed for their ability to sensitize EL4 cells **to** lysis hy E7 49–57 peptide-induced CTL line FaC3 and C3 cell-induced CTL clone 7G1. respectively. Fractions 17 and 18 sensitized EL4 cells for lysis by E7 49–57 peptide-specific CTL line FaC3 (Fig. 1), indicating (hat subdominant epitope E7 49-57 is presented on the cell membrane of C3 cells. Fractions 15 and 16 were recognized specifically by CTL clone 7G1, proving that C3 cells express at least one other CTL epitope besides E7 49-57.

3.4 CTL raised against immuno-subdominant CTL epitope E7 49–57 eradicate HPV16-induced C3 tumors *in vivo*

Finally, we investigated whether CTL line FaC3. raised against subdominant CTL epitope E7 49–57 offered as synthetic peptide, was able to eradicate established C3 tumors *in vivo.* T cell-deficient B6 nude mice with established C3 tumors of about 200 mm^3 were injected weekly with 25 x 10^6 cultured FaC3 cells, administered either i.v. or i.t. in combination with 10^5 IU rIL-2 s.c. As specificity control, a group of mice was included that received Ad5 E1B-specific CTL clone 01 i.t. in combination with 1L-2 s.c. Another group of mice received only IL-2 s.c. In two independent experiments (two and four mice per group, respectively), a total of six out of six mice treated with FaC3 i.t. plus IL-2 s.c. were cured of C3 tumors within 3 weeks (Fig. 2). In the mice receiving IL-2 s.c. only, six out of six tumors grew progressively (data not shown),

This was also observed in each of four mice that were treated with CTL clone 01 i.t. plus IL-2 s.c. (Fig. 2). The latter combination is known to eradicate Ad5-induced tumors ir B6 nude mice [18]. Although *a* tumor growth delay was observed in four of six mice, only one of six mice was cured from its C3 tumor following FaC3 i.v. plus IL-2 s.c. administration (data not shown).

4 Discussion

We have shown that CTL induced against synthetic peptides representing a subdominant epitope recognize the naturally processed counterparts on turnor cells. Expressed on C3 cells, peptide E7 49-57 *h* not immunogenic, inasmuch as CTL induced by O cells recognize another C3 cell-presented peptide instead (Fig. i and Table 1). Whether E7 49–57 is not immunogenic at all when expressed on C3 cells cannot be concluded from the *experiments* shown. Expressed on C3 cells, subdominant epitope E7 49-57 is antigenic, since CTL induced against E7 49–57, necessarily offered as a synthetic peptide, specifically recognize C3 cells (Table 1 and Fig. 1). It is of interest to note that E7 49–57 is immunogenic when presented on H-2^b cells infected with recombinant vaccinia virus expressing HPV16 E7 (24j. Since the E7 protein expression level is probably higher in HPV16 E7-expressing vaccinia virus-infected cells than in HPV16-transformed O cells, the immunogenicity of peptide E7 49–57 is presumably determined by the level of E7 expression. It is known that protein expression levels correlate with the amount of protein-derived peptides that occupy specific MHC class 1 molecules |25j, and that higher expression levels of MHC class I-bound peplidcs are needed for CTL induction (immunogenicity) than for CTL recognition (antigenicity) [26]. We are currently investigating the nature of the immunodominant CTL epitope, the recognition of which is H-2D^b restricted (data not shown). Since C3 cells express at least the E5, E6 and E7 open reading frames ([12] and data not shown), the peptide may be associated with any of these HPV16 proteins.

CTL line FaC3 was induced *in vivo* by E7 49-57 synthetic peptide vaccination, followed by restimulation *in vitro* with E7 49–57 peptide-pulsed RMA-S cells. It cannot be excluded, however, that FaC3 was obtained from primary CTL, induced *in vitro* since it is known that peptide-loaded RMA-S cells are able to induce such a response [26]. After 5 days, the E7 49-57 peptide-induced CTL bulk culture was stimulated with C3 cells instead of with E7 49-57 peptide-pulsed RMA-S (or other syngeneic) cells. This seemed obligatory, since repetitive stimulation with peptide-pulsed cells resulted in the loss of CTL (multiple unpublished observations). We believe that this loss correlates with the concentration of peptide with which the stimulator cells are incubated (unpublished observalions).

CTL directed against immunogenic (dominant) peptides presented at the cell surface of tumor cells are known to eradicate lumors [13, IS. 27, 28]. Our data show, for the first time, that CTL directed against a subdominant CTL epitope can be useful for cancer therapy. These findings are valid for tumor eradication by CTL infusion (this study) as well as for tumor prevention by synthetic peptide vaccination [12]. Peptide-induced CTL may have additional value over whole tumor cell-induced CTL. in the sense that the target peptides do not necessarily need to be immunodominant CTL epitopes. In principle, CTL can be raised against a number of (subdominant) epitopes represented by synthetic peptides, thereby diminishing the chance of CTL escape variants to occur. We have indications that primary synthetic peptide E7 49–57-specific CTL that are induced *in vitro* also eradicate tumors *in vivo* (unpublished observations). Of general importance could be the knowledge that (rumor) cells do nol necessarily need to express immunogenic peptides, to be sensitive to CTL therapy.

CTL line FaC3 eradicated C3 tumors in six out of six mice that received FaC3 i.t., whereas after i.v. administration eradication was observed in only one of six mice. Possibly, when injected i.v. under the conditions used, the number of FaC3 actually arriving at the tumor site was too low to cope with this fast-growing tumor. Only when large numbers of FaC3 were injected directly into the C3 tumor could an adequate E/T radio could be reached *in vivo,* resulting in tumor destruction. Interestingly, the tumor size of the one mouse that was cured from its tumor after i.v. FaC3 administration, was the smallest of all CTL-treated tumors at the start of treatment (60 mm^3). Further experiments are needed to determine whether tumor size is an important factor leading to failure or success of CTL therapy. Alternatively, the homing properties of FaC3 may not be of the same quality as those of previously reported CTL clones that were abïe to eradicate tumors efficiently upon i.v. infusion |J3, 18, 28]. However, absence of homing is unlikely, since CTL with exactly the same specificity as FaC3 can he grown from C3 tumors treated i.v. with FaC3 (preliminary results).

The results presented in this study show, for the first time. that HPV16 E7-specific CTL are directly responsible for the disappearance of HPV16-induced tumor tissue. In another study in mice (C3H), although CTL specificity was not determined, it was suggested that HPV16 E7 acts as a tumor rejection antigen for CTL [29]. Our data are also in agreement with studies showing that a correlation exists between prevalence and progression of HPV(16)-related proliferative disease and impairment of T cell immunity [2–5]. Taken together, these observations justify studies which propose to infuse HPV16-specific CTL in patients with HPV16-induced (pre)malignant lesions. These approaches require the identification of HPV16-derived CTL epitopes that are naturally expressed in the context of human MHC class I molecules [30, 31]. Although our study suggests that tumor eradication is only to be expected following local infusion, preliminary data show that upon therapeutic E7 49–57 peptide vaccination distant from the C3 tumor site, eradication of small lumors (< 50 mm^3) is observed (unpublished results). This indicates that among the induced repertoire, peptide-specific CTL are present lhat do home lo the tumor, suggesting that circulating HPV16-specific CTL are also able to eradicate HPV16-induced tumors.

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Transplantation tolerance induced by antigen pretreatment and depleting anti-CD4 antibody depends on CD4⁺ T cell regulation during the induction phase of the response

Adult mice pretreated with donor-specific transfusion and depleting anti-CD4 antibody 28 days before transplant accept fully allogeneic heart grafts and become specifically tolerant without further treatment. The induction of tolerance in this model is not simply a function of $CD4^+$ T cell ablation, but appears to depend on residual $CD4^+$ T cells which escape depletion and engage donor alloantigen during a transient period of antibody blockade. To test the hypothesis that these $CD4^+$ T cells might be responsible for regulating immune responses toward the graft, mice were reconstituted with naive recipient Leukocytes at various times after pretreatment. Reconstitution either shortly after pretreatment or shortly after transplant had little effect on graft survival. However, when pretreated mice were given an additional dose of depleting anti-CD4 antibody at the time of transplant to target putative regulatory cells, naive leukocytes were able to cause acute graft rejection. These data suggest that in clinical transplantation specific T cell regulation might develop following pretreatment with antigen and non-depleting anti-CD4 antibodies. Such an approach could provide donor-specific unresponsiveness prior to transplant without the risks associated with sustained $CD4^+$ T cell depletion.

1 Introduction

$CD4^+$ T cells play a pivotal role in graft rejection and the breakdown of self-tolerance in the development of autoimmunity. A large number of experimental models have demonstrated that monoclonal antibodies which target this T cell subset can prolong allograft survival or inhibit the onset of autoimmune disease [1–10]. In view of this, anti-CD4 antibodies are attracting increasing attention as clinical therapeutic agents [11–14], but if these reagents are to be used to maximum effect in man, it will be important to establish their precise mode of action.

In murine models, impressive graft prolongation can be achieved using peri-operative administration of antibodies which deplete $CD4^+$ T cells, but in clinical transplantation there may be a reluctance to use such antibodies at the time of transplant because the depletion they cause is profound and long-lasting [13–15]. However, these reagents may have the potential for a much more subtle manipulation of immune responses *in vivo* as shown by the fact that when mice are pretreated with protein antigen under the cover of anti-CD4 antibody, they remain tolerant to the original priming antigen even when the nonspecific effects of the antibody treatment have decayed [16–18]. Tolerance induced by such combined pretreatment is antigen specific and can be maintained by repeated challenge with antigen alone; no further antibody treatment is required. We have translated this approach into a strategy for the induction of tolerance to alloantigen in which recipient mice are pretreated with a donor-specific blood transfusion (DST) under the cover of a depleting anti-CD4 antibody [19, 20]. This pretreatment regimen leads to indefinite cardiac allograft survival in the majority of recipients and is independent of any further immunosuppressive therapy. Graft prolongation is donor specific and leads to operational tolerance in the long-term, in that animals with surviving heart grafts accept donor specific skin grafts but reject those from a third party [20]. We have investigated the mechanisms responsible for allograft survival in this model and demonstrate that the induction of tolerance depends on active immune regulation mediated by $CD4^+$ T cells. Our observations support conclusions which could have an important impact on the way in which the powerful immunosuppressive effects of anti-CD4 antibodies may be exploited in clinical transplantation.

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Abbreviations: DST: Donor-specific blood transfusion **MST:** Median survival time MAR: Mouse anti-rat

Key words: Tolerance / Anti-CD4 antibody / Transplantation

2 Materials and methods

2.1 Mice

Mice (C3H/He; $H-2^k$ and C57BL/10; B10. $H-2^b$) were obtained from Harlan-Olac, Bicester, GB or the National Institute for Medical Research, Harrow, GB.

2.2 Anti-CD4 antibody

The hybridoma YTA 3.1.2 [21] (kindly provided by Prof. H. Waldmann), produces an IgG2b rat anti-mouse CD4 antibody which was prepared as ascites in (LOU x DA)F_1 rats and purified by ammonium sulfate precipitation followed by ion-exchange chromatography. Antibody purity

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