element cycles are already being manifested in high-elevation forests in the eastern United States and Europe (*28*).

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16. Although our data (*12*) and those of G. T. Wolff [*Atmos. Environ.* **18**, 977 (1984)] suggest that airborne particle NO_3^- is dominated by coarse particles, all NO_3^- particle size data must be considered cautiously because of artifacts due to HNO_3 vapor adsorption and NH_4NO_3 volatilization [B. R. Appel and Y. Tokiwa, *ibid.* **15**, 1087 (1981)].
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18. NO_x (NO_2 plus NO) also contributes to the total dry flux of NO_3^-, assuming oxidation in the plant canopy. In a concurrent study at Walker Branch [J. M. Kelly and J. F. Meagher, *Nitrogen Input/Output Relationships for Three Forest Sites in Eastern Tennessee* (Tennessee Valley Authority Air Quality Branch, Muscle Shoals, AL, in press)], NO_x was estimated to contribute an additional NO_3^- dry deposition of 14 mEq/m^2 per year. However, in that study a chemiluminescent NO_x detector was used, and it is not clear to what extent those measurements of NO_x included HNO_3 vapor.
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29. Dry deposition of NH_3 to the forest canopy was estimated by using a deposition velocity for the growing season of 1 cm/sec [published elsewhere (*20*)] and assuming the same ratio of mean concentrations of NH_3 to NH_4^+ (0.05) as measured by those authors. The seasonal and annual mean atmospheric concentrations of NH_4^+ measured in our study and reported by those authors were within 10 percent of each other. Dry deposition during the dormant season was estimated from the growing season value by assuming that the ratio of growing season dry deposition to dormant season dry deposition was the same as that for SO_2 as determined in our study.

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Modification of the Active Site of Alkaline Phosphatase by Site-Directed Mutagenesis

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The catalytically essential amino acid in the active site of bacterial alkaline phosphatase (Ser-102) has been replaced with a cysteine by site-directed mutagenesis. The resulting thiol enzyme catalyzes the hydrolysis of a variety of phosphate monoesters. The rate-determining step of hydrolysis, however, is no longer the same for catalysis when the active protein nucleophile is changed from the hydroxyl of serine to the thiol of cysteine. Unlike the steady-state kinetics of native alkaline phosphatase, those of the mutant show sensitivity to the leaving group of the phosphate ester.

CHEMICAL MODIFICATION AND RECOMBINANT DNA studies directed toward the substitution of active site nucleophiles by other potentially competent nucleophiles have so far been limited to acyl transfer enzymes (*1, 2*). In every case, either the nucleophilic thiol of a cysteine was replaced by the nucleophilic hydroxyl of a serine or the converse substitution was effected. The general result of these modifications was to lower considerably the catalytic activity of the mutated enzymes relative to native enzymes toward all but the most highly activated substrates. The catalytic path of most acyl transfer reactions involves the formation and decomposition of tetrahedral intermediates; since it is the efficiency of these reactions that often dictates the overall enzyme activity, serine-cysteine substitutions may not be well tolerated because of stringent geometric requirements of the active site proton transfer system for a particular molecular environment. Thus, mutation of the active site nucleophile may result in a substantial retardation in the rate of one or more important proton transfer steps.

We now report our observations on the consequences of changing the nucleophilicity of a crucial amino acid side chain at an active site in an enzyme catalyzing another type of group transfer reaction, one that does not require rapid proton transfer in a step affecting the rate. The model system that we investigated is the hydrolysis of phosphate monoesters catalyzed by *Escherichia coli* alkaline phosphatase (BAP) (*3*). The amino acid sequence (*4*) and x-ray map (3.4 Å) of BAP (*5*), a Zn(II) metalloprotein, have been described.

Alkaline phosphatase–catalyzed phosphate ester hydrolysis is initiated by attack of Ser-102 on the phosphoryl group to give a phosphorylated enzyme intermediate that can be isolated at low *p*H (*6*). Subsequently, the phosphorylated enzyme is hydrolyzed and the free enzyme is regenerated. Spectroscopic and kinetic evidence (*7, 8*) indicate that at acidic *p*H the dephosphorylation of intermediate I (Fig. 1) is rate-limiting while

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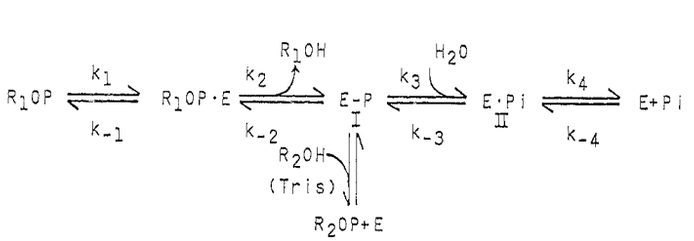
Table 1. Kinetic parameters for the action of BAP and TAP on 4-nitrophenyl phosphate and 2,4-dinitrophenyl phosphate (*15*).

Buffer	Enzyme	4-nitrophenyl phosphate		k_{cat}/K_m (M^{-1} sec^{-1})	2,4-dinitrophenyl phosphate		k_{cat}/K_m (M^{-1} sec^{-1})
		k_{cat} (sec^{-1})	K_m (M)		k_{cat} (sec^{-1})	K_m (M)	
50 mM tris	TAP	4.6	4.1×10^{-5}	1.1×10^5	15	3.5×10^{-5}	4.3×10^5
50 mM NaCl, pH 7.5	BAP	19	5.5×10^{-6}	3.5×10^6	18	5.6×10^{-6}	3.2×10^6
1M tris	TAP	4.3	3.9×10^{-4}	1.1×10^4			
pH 8.0	BAP	73	1.3×10^{-5}	5.5×10^6			



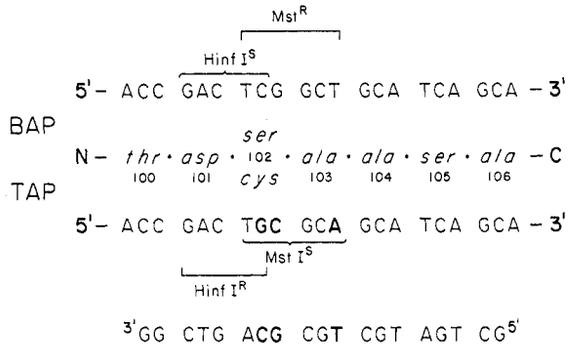


Fig. 1 (left). Scheme for alkaline phosphatase–catalyzed phosphate ester hydrolysis. Both R_1, and R_2 designate either an alkyl or an aromatic group. Fig. 2 (right). DNA coding sequence and amino acid sequences of the active site fragment of BAP and TAP. (Bottom) Primer oligonucleotide used for site-directed mutagenesis.

at alkaline pH the release of phosphate from intermediate II is rate-determining. Whether or not pentacoordinate phosphorus species lie along the pathway for the formation and decomposition of the phosphoryl enzyme is unclear. However, if such pentacoordinate species are formed, they should be very unstable, not requiring proton transfer from an enzyme-bound group for their decomposition.

Thiol alkaline phosphatase (TAP), Ser-102 → Cys-102, was generated by extension of the synthetic oligonucleotide (*9*) shown in Fig. 2. In addition to incorporating base substitutions necessary for changing the wild-type Ser-encoding triplet into one designating Cys, this oligonucleotide was designed to generate a restriction map that is different between the mutant and the wild type. Substitution of the codon specifying Ser (TCG) with that for Cys (TGC) eliminates a Hinf I restriction site, and an additional, silent, change of the third position of the Ala-103 codon (T to A) created an Mst I site.

The template for the mutagenesis was pEMBL9 (*10*) containing a 6.4-kb DNA fragment with the *Escherichia coli phoA* structural gene (*11*). This plasmid contains an origin of replication for f1 phage in addition to its colE1 origin of replication. Single-stranded circular DNA containing the coding strand of the *phoA* gene was obtained when the bacterium harboring this plasmid was superinfected with helper phage (IR-1). The synthetic 19-base oligonucleotide corresponding to the noncoding strand of TAP (Fig. 2) was used to prime DNA synthesis and convert the single-stranded template into double-stranded circular DNA. Mutant plasmids were resolved after two rounds of transformation and identified by colony screening with the same ^{32}P-labeled 19-base oligomer. The presence of the desired mutation was verified by restriction digest studies showing the concurrent appearance of Hinf I resistance and Mst I sensitivity in the plasmid DNA, as well as by direct nucleotide sequencing. The entire protein-encoding region of the TAP gene was sequenced to check that no other mutations had occurred. Except for the desired changes in the DNA sequence encoding Ser-102 to Ala-103, the DNA sequence for TAP is identical to that of the wild-type gene. Because TAP expression levels were low in pT4-1, the original pEMBL9 mutant, the 6.4-kb Pst I fragment containing the *phoA* coding region was transferred into pBR322. The resulting plasmid, pT4391, was then used for TAP expression. The bacterial hosts were *E. coli* strain AW1043 [Δ(*lac*) *galU galK* Δ(*leu-ara*) *phoA*-E15 *proC*::Tn*5*] and its male derivative (F′ *tet*), both of which contain a partially deleted alkaline phosphatase gene, or MPh44[Δ(*phoA-proC*) *phoR tsx*::Tn*5*], which contains no alkaline phosphatase structural gene and is mutant in the *phoR* regulatory gene.

When the *phoR* wild-type strain AW1043 was used as the host for TAP production, the cells were fermented in MOPS medium (*12*) containing 0.1 mM KH_2PO_4 and tetracycline (25 μg/ml) and harvested an hour into the stationary phase. When MPh44, a *phoR* minus strain, was used as the host, the cells were grown under the same conditions but harvested during late log phase. The cells were collected in the presence of phenylmethylsulfonyl fluoride, washed, and treated first with cold hypertonic (sucrose) solution and then with cold hypotonic solution (water). The periplasmic fraction was then isolated and passed through an Affigel Blue (Bio-Rad) column. Alkaline phosphatase is not retained by Affigel Blue and is further purified by ion-exchange chromatography with DEAE-cellulose (15 mM to 200 mM NaCl gradient in 10 mM tris, pH 7.5). The electrophoretic mobility of TAP was identical to that of BAP on sodium dodecyl sulfate (SDS)–reducing polyacrylamide electrophoresis. Gel filtration of BAP and TAP on a Bio-Gel P-150 column indicated that the enzymes are both dimeric, with a molecular weight of approximately 90,000. Kinetic analysis of catalysis by TAP was performed on solutions to which no additional metal ions were added. Although it has been reported that the Zn(II) and Mg(II) are dependent on the action of BAP (*13*), added Zn(II) up to 1 mM did not activate TAP in our experiments.

TAP is an effective catalyst in the hydrolysis of some phosphate monoesters. The most striking difference between the natural Ser-containing enzyme and the Cys mutant is their contrasting selectivity to various substrates. In 1M tris at 10 mM substrate concentration, BAP hydrolyzes the substrates PNPP, 2-naphthyl phosphate, and DNPP at equal rates even though the leaving group tendencies of the alcohols differ greatly. In contrast, the hydrolytic ability of TAP is greatly affected by properties of the leaving group. 4-Nitrophenolate is a better leaving group than 4-methylumbelliferone

and both are superior to 2-naphthol. Consequently, TAP's catalytic efficiency toward PNPP is substantially greater than its action on 4-methylumbelliferyl phosphate and 2-naphthyl phosphate (at 10 m*M* substrate, 1*M* tris (*p*H 8.0), relative rate ratios are 1:0.37:0.06, respectively). This trend is reinforced by the comparisons presented in Table 1; the k_{cat} value of TAP for the hydrolysis of DNPP is three times greater than the corresponding value for the hydrolysis of PNPP, while the k_{cat} values of BAP are nearly identical for both substrates. Although the k_{cat} value of DNPP turnover for TAP is comparable to that for BAP, the rate-determining step may not be equivalent to BAP because TAP is unusually sensitive to the chemistry of the phenolic product. An expected result of the Ser to Cys mutation was the relative shift of the *p*H profile for TAP versus BAP activity (Fig. 3). Under both reaction conditions listed, the *p*H for maximum TAP activity is lower than that for BAP. Since the rates of hydrolysis of PNPP at *p*H 7.5 and 8.0 for each enzyme are quite similar, comparison of the kinetic constants of BAP and TAP at their respective *p*H optima (Table 1) highlights another significant difference between the two enzymes: a fourfold increase of the k_{cat} value for BAP in 1*M* tris versus 50 m*M* tris was in accord with the expected dependence of enzyme activity on buffer concentration. The nucleophilic tris acts as a phosphoryl group acceptor (Fig. 1) (*6*) for the phosphorylated intermediate of BAP, and thus enhances the observed k_{cat} values. In contrast, the k_{cat} value for TAP under the same conditions did not show a dependence on tris. If phosphorylation of the enzyme remains rate-controlling in the presence of tris, then this result would be expected.

All the characteristics of the mutated alkaline phosphatase presented thus far are consistent with the replacement of the wild-type active site nucleophile, serine hydroxyl, by a cysteine thiol. However, the identity of the catalytically important nucleophile came under question when the thiol of TAP's active site could not be easily modified by thiol-directed reagents.

Neither 5,5′-dithiobis(2-nitrobenzoic acid), 6,6′-dithiodinicotinic acid, nor 4,4′-dipyridyl disulfide reacted with TAP as purified from chromatography or after EDTA treatment. The lone free Cys of TAP, not present in BAP, could only be titrated after the enzyme was denatured. When TAP was treated with both EDTA and 6*M* guanidine hydrochloride, a free thiol to polypeptide ratio of 0.97 was determined by titration with 4,4′-dipyridyl disulfide. Furthermore, the mixed disulfide formed between TAP and 4-thiopyridone could be isolated and treated with excess 2-mercaptoethanol to release stoichiometric amounts of 4-thiopyridone. Other compounds unable to modify the active site cysteine of undenatured TAP include 4-chloro- and 4-hydroxymercuribenzoate, Hg(II), iodoacetate, and iodoacetamide.

Steric constraints are probably not the limiting factor for reaction between the thiol of TAP and the modifying reagents since a number of substrates bind to the active site during catalytic turnover. The lack of thiol reactivity can best be explained on the basis of the known x-ray map for the natural enzyme, BAP (*5*). The Ser hydroxyl of BAP is within the ligand binding distance of the active site Zn(II) and, therefore, when the Ser is replaced with a Cys of TAP the thiol may complex the Zn(II). This could suggest that the thiol of TAP may not be the catalytically active nucleophile. Thus, we considered the possibility that TAP does not use the same catalytic mechanism as BAP but might use a carbonic anhydrase–type mechanism in which water ligated to Zn(II) is the crucial nucleophile. The lack of thiol modification in TAP is most likely caused by the proximity of the Zn(II), but this alternative mechanism is definitely not consistent with the following experiment.

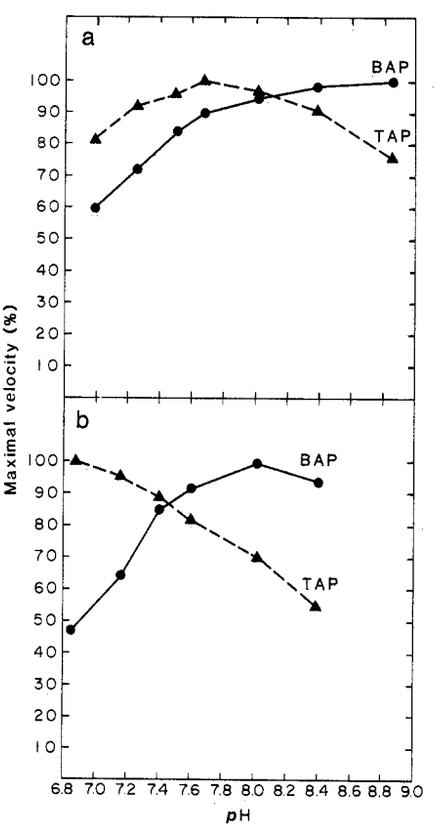


Fig. 3. *p*H dependence of rate of hydrolysis of PNPP at 25°C catalyzed by BAP and TAP at constant substrate concentration. BAP, 0.14 μg in 1 ml of reaction mixture; TAP, 0.7 μg in 1 ml. (a) 50 m*M* tris, 10 m*M* PNPP; (b) 1*M* tris, 1 m*M* PNPP.

Phosphate ester hydrolysis catalyzed by a Zn(II)-water complex would not allow for the transphosphorylation activity of native alkaline phosphatase. For BAP, Ser-102 is clearly the active nucleophile; a phosphoryl-enzyme intermediate can be detected and trapped by acceptors such as tris (*7, 14*). A phosphorylated enzyme intermediate is also evident for the thiol-containing TAP by the effect of tris during catalytic turnover. In the presence of a nonnucleophilic buffer (such as MOPS), TAP catalyzes the hydrolysis of PNPP to produce inorganic phosphate (P_i) and nitrophenolate in a ratio of 1:1 as expected. However, in the presence of increasing concentrations of tris, the amount of P_i released decreases markedly, as it does with BAP (*7*). Since less P_i is formed than nitrophenolate, a substantial degree of transphosphorylation must have occurred to produce a phosphorylated tris derivative. Transphosphorylation catalyzed by TAP would result from a covalent phosphoryl-enzyme intermediate analogous to the one formed during the catalytic cycle of BAP (Fig. 1). The phosphorylated TAP derivative could not be detected directly by precipitating the enzyme in the presence of $^{32}P_i$ at low *p*H.

The observation that the catalytic efficiency of TAP is not affected by tris concentration, but is affected by the characteristics of the phenolic derivative produced during turnover, leads to the hypothesis that the Ser to Cys mutation described above has changed the rate-determining step of catalysis from dephosphorylation or release of P_i to the formation of the phosphoryl-enzyme intermediate. We have shown through our work on alkaline phosphatase that for an enzyme catalyzing a phosphoryl group transfer reaction it is possible to change the active site nucleophile and still observe high k_{cat} values.

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15. Substrate activation was observed for BAP at concentrations greater than $5 \times 10^{-4} M$ PNPP in tris at low ionic strength. Below this value a linear relation was observed for the Lineweaver-Burk plot. At 1*M* tris the plot is linear over a wide substrate range. In contrast to the behavior of BAP, TAP shows no substrate activation.
16. We thank Drs. H. Inouye and J. Beckwith for providing the original alkaline phosphatase plasmid and *E. coli* strains used in this work; Drs. D. Kendall, P. Model, and M. Russel for advice; and J. F. Harris, J. Marrinan, D. Levitan, K. Howard, and E. Radziejewski for technical assistance. Supported in part by a postdoctoral fellowship AM 07122-02 (S.E.R.) from the National Institutes of Health. This report is dedicated to the memory of Dr. Kazys Martinkus.

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Specific Immunosuppression by Immunotoxins Containing Daunomycin

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Daunomycin, when conjugated with a targeting antigen by an acid-sensitive spacer, remains inactive at the intravascular *p*H of 7 but becomes active after cleavage within the acidic lysosomal environment of the target cell. This observation made it possible to construct cytocidal compounds that caused antigen-specific suppression of murine lymphocyte function. When daunomycin was coupled to the hapten conjugate of ovalbumin by an acid-sensitive *cis*-aconityl group, it caused hapten-specific impairment of immunocompetence in murine B lymphocytes in vitro and in vivo. Furthermore, the response by T lymphocytes to concanavalin A in vitro was selectively eliminated by a conjugate between daunomycin plus the acid-sensitive spacer and a monoclonal antibody specific for T cells.

THE SPECIFIC TARGETING OF CYtocidal drugs to selectively eliminate undesirable cells has met with only limited success, particularly in vivo (*1*). Recently, various toxins, including ricin, have proved to be specifically cytotoxic in vitro and, in a few cases, in vivo after conjugation to antibodies raised against cell surface markers (*1–4*). The clinical potential of immunotoxins includes the immunotherapy of cancer and the alleviation of various immunoregulatory disorders and allograft rejection. For example, clones of B lymphocytes may be selectively eliminated by the specific binding of the antigen-toxin conjugate to cell surface immunoglobulin receptors. Such studies have involved the specific deletion in vitro of B lymphocytes by ricin that had been conjugated to antigens, including self antigens such as acetylcholine receptors and thyroglobulin (*5–7*).

Unfortunately, the use of toxic peptides such as ricin raises the serious problem of nonspecific toxicity, a formidable obstacle for application in vivo (*4*). For this reason, we have used daunomycin (DM) as a cytocidal drug in conjunction with a targeting hapten-coupled protein (*8*). When directly linked to a protein carrier by reaction with an amino group, DM tends (in some cases) to lose its cytocidal potential (*9*). This problem was overcome through the introduction of an acid-sensitive spacer such as a *cis*-aconityl group (CA) between carrier and drug (*10*). The carrier–(CA-DM) conjugate remained stable at the intravascular *p*H of 7 but once endocytosed by the target cell, the drug was cleaved in the intralysosomal acid environment and entered into the cytoplasm. Intercalation of DM appears to make template DNA unavailable to DNA and RNA polymerases (*11, 12*).

We report here that trinitrophenol (TNP)-, 4-hydroxy-3-iodo-5 nitrophenylacetic acid (NIP)-, and fluorescein (FLU)-ovalbumin (OVA) conjugates, when coupled to CA-DM, are capable of inducing hapten-specific immunosuppression in murine lymphocytes in vitro and in vivo. We also demonstrate that conjugates between a T-cell–specific monoclonal antibody (anti-Thy 1.2) and CA-DM selectively suppress the proliferative response by murine spleen cells to the T-cell mitogen concanavalin A (Con A). This effect probably reflects the selective binding of the drug-antigen conjugate to target B or T lymphocytes bearing surface receptors for the hapten or anti-Thy 1.2, respectively.

To prepare CA-DM, a modified version of the method of Shen and Ryser (*10, 13*)

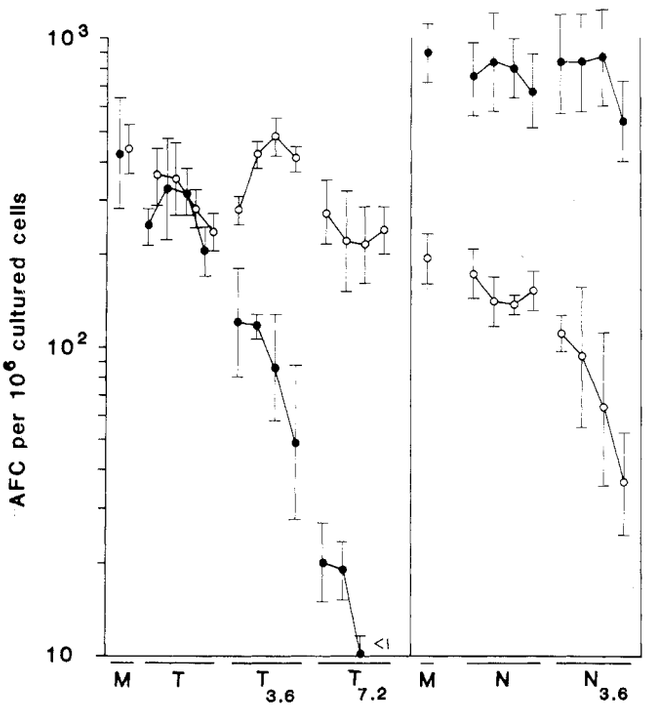

Fig. 1. TNP- or NIP-specific immunosuppression induced in vitro with TNP-OVA-(CA-DM) or NIP-OVA-(CA-DM). In each group, interconnected symbols represent treatment of splenocytes (4×10^6 cells per milliliter) for 2 hours at 4°C with the indicated conjugate at 50, 100, 250, and 500 μg/ml. M, medium alone; T, TNP_7-OVA; $T_{3.6}$, TNP_7-OVA-$(CA\text{-}DM)_{3.6}$; $T_{7.2}$, TNP-OVA-$(CA\text{-}DM)_{7.2}$; N, NIP_3-OVA; $N_{3.6}$, NIP_3-OVA-$(CA\text{-}DM)_{3.6}$. The treated cells were washed and challenged with TNP-Ba (closed circles) or NIP-Ficoll (open circles) in vitro for 4 days. Mean numbers of antibody-forming cells (AFC) ± standard deviation are indicated on the ordinate.

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