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The cytolytic T lymphocyte response to the murine cytomegalovirus
II. Detection of virus replication stage-specific antigens by separate populations of *in vivo* active cytolytic T lymphocyte precursors*

During the acute cytolytic T lymphocyte (CTL) response of mice to infection with the murine cytomegalovirus two independent populations of activated interleukin-receptive CTL precursors can be demonstrated. One population is specific for cell membrane-incorporated viral structural antigens, whereas the second population detects an antigen, whose appearance is correlated with the synthesis of viral immediate early proteins. Since this new type of antigen is only defined by lymphocyte recognition, it is referred to as the lymphocyte-detected immediate early antigen (LYDIEA). Expression of immediate early antigen precedes the production of viral progeny and, therefore, it is possible that LYDIEA-specific CTL could serve as indicator cells for the very first activities of the viral genome, even during nonproductive infection.

1 Introduction

The observation that membrane insertion of purified viral envelope glycoproteins is sufficient to render target cells susceptible to lysis by virus-specific CTL [1] has initiated a series of reports on the capacity of CTL to discriminate between cells infected with serologically related or unrelated viruses (for review see [2]). In the majority of systems studied, it appears that the CTL response is less specific than the antibody response.

Concerning a physiological role of CTL in the defense against a particular host-specific pathogenic virus, the relevance of this "type specificity" is limited if virus strains, which do not infect the same host naturally, are compared. A more promising approach might be to investigate whether CTL can discriminate between different antigens specified by the same virus and expressed at different stages of the viral replication cycle. Herpes virus infections allow a dissection into distinct stages and thus can serve as model systems to test the "replication stage specificity" of CTL. Productive infection with murine cytomegalovirus (MCMV) is analogous to that described for herpes simplex viruses [3, 4] and leads to a coordinately regulated synthesis of viral proteins which can be

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Abbreviations: **Act.D:** Actinomycin D **CH:** Cycloheximide **CTL:** Cytolytic T lymphocytes **ICP:** Infected cell protein(s) **I.E.:** Immediate early **IL-CTLp:** Interleukin-receptive CTL precursors **LYDIEA:** Lymphocyte-detected I.E. antigen(s) **MCMV:** Murine cytomegalovirus **MEF:** Mouse embryo fibroblast(s) **PFU:** Plaque-forming units **p.i.:** Post infection **LN:** Lymph node(s)

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arranged in at least three groups, denoted alpha (immediate early, I.E.), beta (early) and gamma (late). I.E. proteins are defined operationally as the first viral proteins translated in the presence of actinomycin D (Act.D) after removal of the protein synthesis inhibitor cycloheximide (CH). Under these experimental conditions the synthesis of early and late proteins is effectively prevented and thus also the appearance of the major viral structural proteins.

In a preceding report, we have analyzed the events in a draining lymph node (LN) during acute MCMV infection and have introduced interleukin-receptive CTL precursors (IL-CTLp) as representatives of the *in vivo*-active fraction of the specificity repertoire [5]. Since these cells are not influenced by the selective effects of antigen stimulation *in vitro*, they can be used as a probe to define the identity of those antigens which give rise to an antiviral CTL response. In the present report, we demonstrate that functionally defined IL-CTLp comprise at least two distinct specificity classes. One population of IL-CTLp-derived CTL exerts lytic activity towards cells carrying viral structural proteins. A second population detects antigen(s) whose occurrence is correlated with the expression of I.E. proteins. This type of antigen(s) is so far only defined by lymphocyte recognition and is therefore designated lymphocyte-detected immediate early antigen (LYDIEA).

2 Materials and methods

2.1 General remarks

The generation of MCMV-specific CTL, virus preparation, cytolytic assay and statistical calculations have been described in a preceding report [5]. Female BALB/c (H-2^d) mice were used as responders.

2.2 Preparation of I.E. target cells

Secondary passage mouse embryo fibroblasts (MEF) were infected as preconfluent monolayers under the influence of a centrifugal field (800 × g for 30 min). The centrifugation step leads to an enhancement of infectivity [6, 7] and the resulting multiplicity of infection is about 20–80-fold the original dose of infection. Because of the uncertainty in estimating the factor of enhancement, the dose of infection (plaque-forming units, PFU/cell) as determined in a conventional plaque titration assay is given in the figures. Infection and 3 h of cultivation were carried out in presence of CH (50 μg/ml; Sigma, Taufkirchen, FRG) to prevent protein synthesis. Thereafter, CH was washed out quantitatively and the monolayer was cultured for further 2 h in the presence of Act.D (5 μg/ml; Sigma). Finally the cells were suspended, labeled with ^{51}Cr and used as targets in the cytolytic assay. All these steps were performed under Act.D to prevent transcription. UV inactivation of virus for preparation of structural antigen target was performed with an UV illuminator at 254 nm for 5 min (RU-VE chroma 41, Vetter, Wiesloch, FRG).

2.3 Preparation of MCMV-specific antiserum

Female BALB/c mice at 4–6 weeks of age were infected 3 times i.p. with 10^5 PFU of partially purified MCMV, at intervals of 2 weeks. The serum taken 14 days after the last boost served as immune serum.

2.4 Immunoprecipitation analysis

Biosynthetic labeling of infected MEF was performed after withdrawal of CH in methionine-free minimum essential medium containing 5% fetal calf serum and 1.25 mCi = 46.25 MBq/ml [^{35}S]methionine (Amersham, Braunschweig, FRG) and 5 μg/ml Act.D for 4 h. Thereafter, the cells were lysed by sonication in extraction buffer (10 mM Tris-HCl, pH 7.5 containing 150 mM NaCl, 1% sodium deoxycholate, 1% Nonidet-P40, 0.1% sodium dodecyl sulfate (SDS), 2 mM L-methionine, 1 mg/ml ovalbumin and 1 mM phenylmethylsulfonyl fluoride) and the lysates were centrifuged at 40000 rpm (60 min, 4 °C) in a Beckman 50 Ti rotor. Supernatant aliquots of 1×10^6 cpm were incubated (60 min at 20 °C and then 60 min on ice) with 0.01 ml antiserum or normal mouse serum, and the immune complexes were precipitated by the addition of 50 μl *Staphylococcus aureus* (Pansorbin, Calbiochem, Giessen, FRG). The precipitates were washed and then solubilized in sample buffer (62.5 mM Tris-HCl, pH 6.8, 3% SDS, 10% sucrose, 5% 2-mercaptoethanol, 0.01% bromophenol blue) at 95 °C for 5 min and analyzed in 10% SDS polyacrylamide slab gels [8]. Gels were processed for fluorography with En^{3}Hance (New England Nuclear, Boston, MA) and bands were visualized with Kodak x-Omat R films.

3 Results

3.1 Detection of newly synthesized antigens is infection-dose dependent

To define the virus-encoded or virus-induced membrane antigens, which are synthesized in the course of the viral replication cycle, it is mandatory to determine a possible contribution

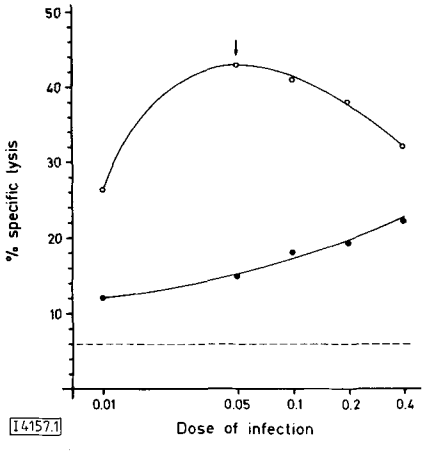

Figure 1. Dose dependence of target formation. Day 8 immune lymphocytes (BALB/c, 10^5 PFU MCMV footpad, popliteal LN) were plated at a density of 10^5 cells/microwell. CTL obtained after 6 days of IL-mediated (20%, v/v) expansion in the absence of antigen were tested on syngeneic MEF, which were either infected and incubated for 20 h with various doses of MCMV (○—○) or in parallel mock-infected with UV-inactivated virions (●—●). Infection was performed under centrifugal enhancement conditions (800 × g, 30 min) to synchronize the viral replication. The dose of infection is given in PFU/cell. The median values (n = 24) of lytic activity are depicted. The dashed line indicates the detection level belonging to the optimum condition (arrow).

of those viral structural proteins to target cell formation which could be integrated into the cell membrane by fusion during the initial steps of infection. The data shown in Fig. 1 demonstrate that lytic activity towards cells loaded with input antigens increased with increasing doses of UV-inactivated virions.

If infectious virus was used and viral replication was allowed to proceed for 20 h, "late" conditions were obtained characterized by production of viral DNA and structural protein synthesis. This way of producing target cell formation was most effective and generated optimal assay responses (arrow in Fig. 1) at a relatively low dose of infection. At higher doses, the susceptibility of late targets to CTL-mediated lysis decreased for unknown reasons, until finally, the influence of newly synthesized antigens and input antigens could no longer be distinguished. In repeated experiments the optimum conditions varied to some extent depending on the quality of the individual MEF batches used for target cell preparation. For this reason it was necessary to control for input antigens in each experiment.

3.2 CTL detect an antigen in parallel to the expression of viral I.E. proteins

Having established the conditions for detecting newly synthesized antigens, the identity of these antigens and the temporal pattern of expression could be analyzed. The sequence A–D in Fig. 2 illustrates the kinetics of target cell formation during productive infection. In this case antigens were first detectable by CTL under "early" conditions at 12 h post infection (p.i.) (C), *i.e.* before the onset of viral DNA replication at

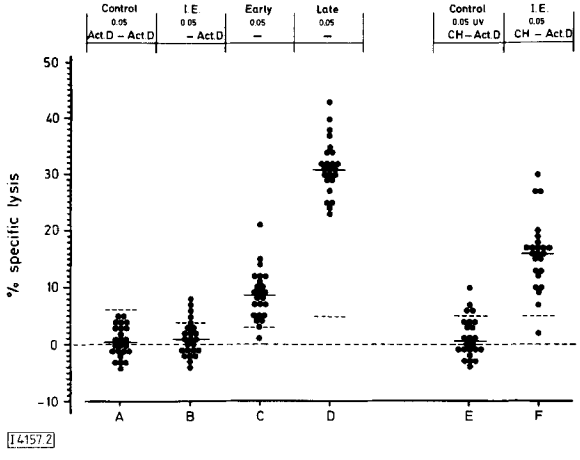

Figure 2. Kinetics of target formation and enhancement of LYDIEA expression by treatment with metabolic inhibitors. MCMV-specific CTL were generated as outlined in the legend to Fig. 1 and tested on the different target cells indicated. The dose of infection was 0.05 PFU/target cell throughout. The rationale for the various inhibitor combinations is given in Sect. 3.2. The treatment with metabolic inhibitors can be dissected into 2 steps. The first step includes infection and the first 3 h of cultivation. The second step lasts until the end of the assay period. Under "early" conditions (C) the viral replication in target cells did not exceed 12 h, including the assay period, whereas target cells expressing late antigens (D) were infected at least 20 h before entering the lytic assay. The dashed horizontal bars indicate the 99% tolerance limits of the low control normal distributions (n = 24) and the solid horizontal bars mark the median values of the experimental data. (n = 24).

16 h p.i. (A. Ebeling, unpublished observations). The highest lytic activity was measured on "late" targets at 20 h p.i. (D). If the ongoing of infection was blocked by Act.D as early as 3 h p.i., antigens were not detectable by CTL (B). This failure could reflect either an insufficient expression of antigens on these cells or a lack of CTL specific for the antigens expressed.

To decide between these alternatives a special experimental device was applied: if protein synthesis is inhibited by CH during infection and for several hours thereafter, only a limited number of viral genes can be transcribed by the cellular RNA polymerase II [9]. These genes, the corresponding transcripts and their translation products have been designated "I.E." [4]. In the normal course of infection, the synthesis of I.E. proteins is regulated and only transient. Cessation of this regulation as a consequence of CH treatment causes an accumulation of I.E. messenger RNA. Removal of CH and addition of the transcription inhibitor Act.D then allows enhanced synthesis of I.E. proteins and simultaneously prevents the appearance of early and late transcripts [4].

It is noteworthy that this enhancement effect was not only demonstrable on the level of the I.E. proteins (compare lanes E and F, Fig. 4) but also on the level of the CTL-target cell interaction (compare panels B and F, Fig. 2).

In conclusion, this finding excludes the second alternative and indicates that I.E. antigens render target cells susceptible to lysis, provided they are expressed in sufficient amounts (F).

As outlined in Sect. 3.1, it is mandatory to control for input antigens. In the present experiment only newly synthesized antigens were detected, since lysis was not observed on target cells mock-infected with UV-inactivated virions (E) or when after infection with the same dose of virus transcription was blocked by Act.D throughout the target formation and assay period (A). The data of the experiment shown in Fig. 3 support and extend these results under modified conditions. As a consequence of the increased dose of infection, input antigens were now detected. The level of lytic activity was comparable irrespective of whether the target cells were prepared with UV-inactivated virions (B) or whether transcription (C) or translation (D) were blocked during infection. These findings confirm that target formation is possible without *de novo* synthesis of proteins, but also imply that the expression of I.E. antigens [compare (A) to (B–D)] indeed requires infectious virus [compare (A, B)], transcription [compare (A, C)] and translation [compare (A, D)]. The presence of CH during the assay period after completed antigen synthesis did not cause significant inhibition of target cell lysis [compare (E, F)].

The synthesis of I.E. proteins during the period of target formation was studied in parallel to the cytolytic tests to control the differences between the target cell preparations (Fig. 4). In contrast to infection with herpes simplex viruses [4] cellular protein synthesis is not switched off during infection with MCMV [10]. Immunoprecipitation was therefore used to detect virus-encoded or virus-induced proteins in infected cells (ICP). Lanes A–C demonstrate the influence of the infection dose on the rate of I.E. protein synthesis. Three predominant I.E. polypeptides were precipitated (manuscript in preparation). Lanes D–F document the CH-mediated enhancement of the amount of I.E. protein. After mock-infection with UV-inactivated virions ICP were not detectable (compare lanes D and F), indicating that the inactivation was effective. This once

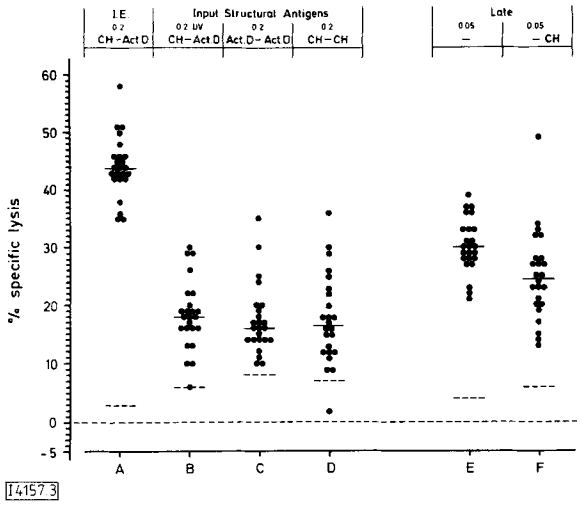

Figure 3. LYDIEA expression requires infectious virus, transcription and translation. BALB/c MEF were infected with 0.2 PFU MCMV/cell and different combinations of inhibitors were applied to allow or prevent I.E. antigen expression (A–D). Late antigen expression (E–F) was obtained by infection with 0.05 PFU/cell followed by 20 h of viral replication. Target cells F were further incubated in the presence of CH identical to the treatment of target cells D.

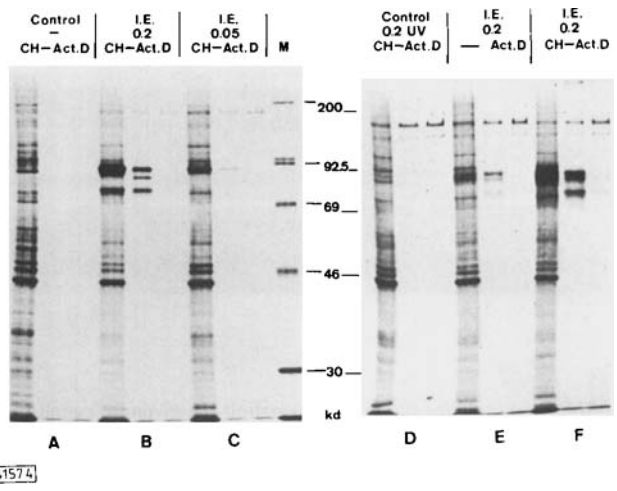

Figure 4. Rate of protein synthesis in MCMV-infected MEF under different immediate early conditions. I.E. protein synthesis in the indicated target cell modifications was analyzed by immunoprecipitation. The first lane in each triplicate group represents the cell lysate with all polypeptides synthesized immediately after removal of CH. The second lane shows those polypeptides which could be precipitated with BALB/c MCMV-specific immune serum and as a specificity control the third lane documents the precipitation with autologous preimmune serum.

again supports the conclusion that the lytic activity observed in panel B of Fig. 3 was not directed towards *de novo* synthesized ICP.

3.3 I.E. antigens and viral structural antigens are recognized by different populations of CTL

So far the experiments have established that a LYDIEA exists and can be demonstrated as a separate entity by the use of an appropriate target cell preparation, which lacks detectable amounts of viral structural antigens [compare (E) and (F), Fig. 2]. This by itself does not yet exclude the possibility that LYDIEA is part of the virion and accounts for the lytic activity observed on target cells prepared with inactivated virus. To test this possibility individual microcultures, set up with MCMV-primed LN lymphocytes, were split into four identical fractions after a 6-day antigen-free expansion period and assayed twice on I.E. target cells and twice on target cells carrying viral structural antigens. As evaluated by Spearman rank correlation analysis (Table 1) the lytic activities on the two types of target cells were independently distributed and thus the targets under test evidently did not share antigens relevant for recognition by CTL. These observations allow the conclusion that, in response to infection with a particular virus, distinct populations of CTL arise which recognize different antigens in a specific way. Phenotype analysis revealed that both populations can be characterized as Thy-1$^+$, Lyt-2$^+$ lymphocytes (data not shown).

3.4 During infection LYDIEA-specific memory CTL are induced

Lymphocytes from different somatic or visceral lymph nodes of 16-month-old mice infected neonatally with MCMV or of age-matched noninfected controls were subjected to IL-mediated expansion in presence or absence of viral antigen (Table 2). Results show that lymphocytes from normal mice cannot be activated to lyse cells expressing LYDIEA or late viral antigens and that generation of cytolytic effector cells *in vitro* requires specific memory and antigen, but not interleukin only. This fact documents the absence of IL-CTLp in these LN at that stage of infection and also excludes that natural killer (NK) cells which could perhaps lyse I.E.-infected cells in a nonantigen-specific manner are generated by IL *in vitro*. Depending on the LN they were derived from, the restimu-

Table 1. LYDIEA and viral structural antigens are recognized by separate populations of CTL[a)]

Target comparison		R_s	p	Correlation hypothesis
I.E. 1	– I.E. 2	0.671	0.0006	Accepted
S 1	– S 2	0.650	0.0009	Accepted
I.E. 1	– S 1	–0.073	0.3632	Rejected
I.E. 2	– S 2	–0.129	0.2676	Rejected
I.E. 1	– S 2	–0.153	0.2327	Rejected
I.E. 2	– S 1	0.072	0.3632	Rejected

a) Replicate cultures (n = 24) were set up with 10^5 lymphocytes at day 8 after priming with 10^5 PFU MCMV. After a 6-day *in vitro* expansion period the individual microcultures were split 4-fold and tested for lytic activity twice (groups I.E. 1 and I.E. 2) on I.E.-MEF (0.05 PFU/cell, CH-Act.D) and twice (groups S 1 and S 2) on S-MEF loaded with structural antigens (4 PFU MCMV, UV-inactivated/cell). The individual cultures were ranked according to their lytic activity and the Spearman rank correlation coefficient (R_s) was calculated [11]. For $R_s \times (n-1)^{0.5}$ the attached probability values p were taken from tables of the standard normal probability function [12]. The correlation hypothesis was accepted for positive R_s and $p < 0.05$ (one-tailed test).

Table 2. Detection of memory cells specific for LYDIEA or late antigen in latently infected mice[a)]

MCMV proteins expressed by target cells	Mesenteric LN		Axillary LN	
	Expansion	Restimulation	Expansion	Restimulation
Late[b)]	< DL[c)]	37.5 (17.3–67.0)[d)]	< DL	33.9 (18.5–46.0)
I.E. (CH-Act.D)	< DL	29.4 (5.1–45.2)	< DL	11.9 (0.6–25.3)

a) Newborn BALB/c mice were infected i.p. with 10^2 PFU of MCMV. In surviving females, a latent infection was established after 6–8 months. After 16 months mesenteric as well as axillary LN were tested for active IL-CTLp by expansion of 10^5 lymphocytes for 6 days in the presence of IL alone or for memory CTL by *in vitro* restimulation in the presence of IL and, in addition, 0.2 PFU/cell infectious virus as antigen. Lytic activity measured on mock-infected MEF never exceeded the detection level (not included in the Table). In neither of the protocols could MCMV-specific activity be detected when lymphocytes of noninfected age-matched control mice were tested.

b) The conditions for target cell formation are given in legend to Fig. 2.

c) Minimum positive detection level, defined by the 99% tolerance limit of the low control (n = 24) that ranged from 3.9 to 6.2% specific lysis.

d) Median value (range) % specific lysis determined from n = 24 individual microcultures.

lated cells showed different ratios of lytic activity towards the two types of infected target cells. This finding is indicative for a nonrandom distribution of antigen-specific memory T cells in different lymphoid organs.

4 Discussion

This report is part of a study on the T cell response of mice to infection with the MCMV. Three observations have been made: first, target cells could be prepared that carried either only viral structural proteins or expressed only I.E. proteins. An analysis of the CTL response with respect to these target cell properties allowed the conclusion that ICP synthesized during the I.E. phase of infection were sufficient to render cells susceptible to CTL-mediated lysis. This new type of antigen(s) has been termed LYDIEA. Secondly, CTL recognizing LYDIEA and CTL specific for viral structural antigens belong to different populations. Thirdly, IL-CTLp with specificity for these different antigens coexist *in vivo* as active cells at the peak of the acute immune response. Earlier reports on pox viruses [13–17] and rhabdo viruses [18] have already addressed the question of whether early virus-induced membrane alterations can be recognized by CTL. Using virus mutant strains [13, 14] or inhibitors of protein synthesis [16–18], it was concluded that early antigen expression preceding viral genome replication and virion assembly is sufficient for target cell formation. The question, however, of whether recognition of different antigens may occur was not approached in these studies. Experiments on the immune response to herpes viruses led to contradictory interpretations, because the contribution of input viral structural antigens could not be evaluated [19–21]. This question can now be answered. Distinct classes of antigens, corresponding with the I.E. and the late phase of the MCMV replication cycle, can be identified by separate populations of CTL that appear to be "replication-stage specific". Thus, CTL can discriminate between different antigens induced by one virus. This is in remarkable contrast to the frequent observation that CTL cannot discriminate even between serologically distinguishable substrains of myxo and rhabdo viruses [2]. It is possible that antigens specified by different virus substrains, but expressed at the same replication stage, are antigenically more related than antigens specified by one virus but expressed at different replication stages.

An objection to the *in vivo* relevance of LYDIEA expression could be that significant lysis of cells was only obtained after enhancement of I.E. protein synthesis which, under permissive conditions *in vitro*, is only low and transient [3]. The fact, however, that the IL-CTLp which recognize LYDIEA represent *in vivo* active cells not depending on further *in vitro* restimulation and selection by antigen provides evidence that LYDIEA expression *in vivo* is sufficient to induce a clearly demonstrable acute response. In addition, specific memory CTL for LYDIEA were generated. This indicates either that I.E. ICP are highly immunogenic or, more likely, that expression of I.E. antigens *in vivo* is more abundant than expected from studying productive infections *in vitro*. These alternatives are presently under investigation. If the latter is correct, IL-CTLp may serve as indicator cells for the intensity of viral antigen expression *in vivo*. The antigen LYDIEA is characterized as an ICP synthesized under I.E. conditions and detected by lymphocytes. Thus far there is no evidence for an identity with any of the I.E. proteins detected by immunoprecipitation and there is also no proof that LYDIEA is encoded by the viral genome. The situation is reminiscent of the lymphocyte-detected membrane antigen (LYDMA) which is seen by T cells on Epstein-Barr virus-transformed B cells, but could not be identified as a viral product ([22], reviewed in [23]).

The existence of CTL clones selectively recognizing LYDIEA was so far only shown on the population level. The preparation of LYDIEA-specific CTL clones would give additional proof for these findings, but selection of such clones by limiting dilution is difficult considering the multitude of MCMV virus structural proteins [24] and the paucity of I.E. proteins (Fig. 4). Antigen-specific selection was so far not possible due to the toxicity of I.E.-infected inhibitor-treated cells. To express selectively I.E. proteins in mammalian cells the MCMV genome was cloned [25] and the genomic regions coding for I.E. RNA were mapped (G. Keil et al., submitted for publication). Presently, appropriate subclones to be inserted into a shuttle vector [26] are under construction.

IL-CTLp appear to be useful for analyzing the antigen specificity of the antiviral CTL response. Furthermore, if they exist beyond the acute infection, the LYDIEA-specific subpopulation can serve as an indicator for persistent or recurrent activity of the MCMV genome. This activity might even be detected under conditions where the viral replication cycle

does not proceed to the production of viral progeny and thus IL-CTLp may provide a new tool to study herpes virus latency.

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Ontogeny of surface markers on functionally distinct T cell subsets in the chicken*

Three subsets of chicken peripheral T cells (T1, T2 and T3) have been identified in peripheral blood of adult chickens on the basis of fluorescence intensity after staining with certain xenogeneic anti-thymus cell sera (from turkeys and rabbits). They differentiate between 3–10 weeks of age in parallel with development of responsiveness to the mitogens concanavalin A (Con A), phytohemagglutinin (PHA) and pokeweed mitogen (PWM). Functional tests on the T subsets, sorted with a fluorescence-activated cell sorter, have shown that T2,3 cells respond to Con A, PHA and PWM and are capable of eliciting a graft-*vs.*-host reaction (GvHR). In contrast, although T1 cells respond to Con A, they respond poorly to PHA and not at all to PWM or in GvHR. There was some indication of cooperation between T1 and T2,3 cells for the PHA response. Parallels between these chicken subsets and helper and suppressor/cytotoxic subsets in mammalian systems are discussed.

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Abbreviations: **ABS:** Anti-bursa cell serum **ATS:** Anti-thymus cell serum **Con A:** Concanavalin A **FACS:** Fluorescence-activated cell sorter **FITC:** Fluorescein isothiocyanate **GvHR:** Graft-*vs.*-host reaction **LAF:** Lymphocyte-activating factor **mAb:** Monoclonal antibody(ies) **PBL:** Peripheral blood leukocytes **PBS:** Phosphate-buffered saline **PHA:** Phytohemagglutinin **PWM:** Pokeweed mitogen **TRITC:** Tetramethylrhodamine isothiocyanate

1 Introduction

The chicken immune system has been much less extensively characterized than that of the mouse or man, but several lines of evidence have indicated that immunoregulatory circuits similar to those found in mammalian systems also exist in this species. Amplifier and suppressor functions have been described in several different test systems [1–13] and recently the production of chicken interleukins 1 and 2 has been described [14, 15].

For formal characterization of T helper and suppressor/cytotoxic subsets and study of their interactions, in a similar manner to that originally described in the mouse system

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