Matthias Johannes Reddehase, James Hilton Cox and Ulrich Helmut Koszinowski

Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen

Frequency analysis of cytolytic T cell precursors (CTL-P) generated *in vivo* during lethal rabies infection of mice
I. Distinction of CTL-P with different interleukin 2 sensitivity*

The aim of this study was to determine the number and state of activity of cytolytic T lymphocytes (CTL) and their precursors (CTL-P) present *in vivo* during the early stages of viral infection. The local response to lethal infection with rabies virus was used as a model system that is not accessible to analysis by secondary activation *in vitro*. The local response to alloantigen served as a control. Experimental protocols were established that allow frequency estimates of *in vivo* antigen-triggered CTL-P. Data allow a distinction between CTL-P activated *in vivo* by alloantigen and viral antigen with respect to their different capacity to utilize T cell growth factors (interleukins). *In vivo* alloantigen-primed CTL-P generate, *in vitro*, an active effector progeny in the presence of interleukins of xenogeneic origin, whereas the majority of virus-specific CTL-P, in spite of considerable expansion *in vivo*, fail to generate CTL *in vitro* unless antigen is added.

1 Introduction

Cell-mediated immunity appears to play a role in host control of rabies virus infection. In mice, a cytolytic T cell (CTL) response is induced only by attenuated rabies virus strains, but not after infection with virulent strains causing lethal disease. Thus, the level of T cell response may predict the course of the murine infection [1–4]. Data reported so far were obtained in long-term (16–24-h) assays not directly comparable with data in the conventional 4-h assay and contain little quantitative information. In this report, the first frequency estimates of rabies-specific cytolytic T lymphocyte precursors (CTL-P) and their *in vivo* generated interleukin-sensitive progeny (IL-CTL-P) are described in direct comparison with the corresponding frequencies observed in the response towards allogeneic major histocompatibility complex (MHC)-encoded antigens under 4-h assay conditions. The determination of CTL-P frequency was carried out by different experimental protocols. The experiments were designed to obtain information on the activity and frequency of cells triggered by antigen *in vivo*. Therefore, culture conditions were established that avoided the selective effects of secondary exposure of CTL-P to antigen *in vitro*.

We find that even during a lethal rabies infection specifically sensitized T cells are present and can be expanded *in vitro* to detectable cytolytic progeny in the presence of T cell growth factors (IL 2). Interestingly, after *in vivo* sensitization by antigen, the majority of alloantigen-specific CTL-P are susceptible to the effect of IL 2 *in vitro*, whereas most of the cells activated by rabies virus need further stimulation by antigen *in vitro* to generate functionally active clones. It may be this deficiency or delay in maturation that limits the potential protective role of cell-mediated immunity during a lethal rabies infection.

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Correspondence: Ulrich H. Koszinowski, Institut für Immunologie, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Paul-Ehrlich-Str. 28, P.O. Box 1149, D-7400 Tübingen, FRG

Abbreviations: **CTL:** Cytolytic T lymphocytes **CTL-P:** Cytolytic T lymphocyte precursors **ERA:** Rabies virus, strain ERA **IL:** Interleukin **IL-CTL-P:** IL-receptive CTL-P **LN:** Lymph node(s) **SC:** Spleen cell(s) **MHC:** Major histocompatibility complex

2 Materials and methods

2.1 Mice

C57BL/6 (H-2^b) and DBA/2 (H-2^d) mice were purchased from the Zentralinstitut für Versuchstiere, Hannover, FRG. Male mice were used at 4–6 weeks of age.

2.2 *In vivo* priming for CTL effector cell responses

C57BL/6 (H-2^b) mice were primed by footpad injection of either 5×10^6 mitomycin C-treated DBA/2 (H-2^d) spleen cells (SC) or 10^7 plaque-forming units (PFU) of gradient-purified tissue culture (BHK)-grown ERA strain of rabies virus (ERA). Mice were killed four days after antigen application.

2.3 Microcultures

The cells of the draining popliteal lymph nodes (LN) were suspended in RPMI 1640 medium (2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10% v/v fetal calf serum) and graded numbers in 36 replicates/concentration were plated in 96-well round-bottom microtiter plates (Greiner, Nürtingen, FRG). Incubation was performed under standard conditions (37 °C, 5% CO_2, humidified atmosphere) for 9 days in the

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presence of mitomycin C-treated stimulator (5×10^5 DBA/2 SC, 10^4 ERA-infected MC57G (H-2^b) fibrosarcoma cells) or filler cells (5×10^5 C57BL/6 SC, 10^4 noninfected MC57G cells) depending on the protocol intended. ERA infection of cells was performed 24 h prior to use and efficiency of infection was controlled by direct immunofluorescence. Cultures were supplemented with 40% v/v IL 2-containing supernatant obtained from concanavalin A (Con A)-activated rat SC. Suppressive factors in the supernatant were eliminated by 24 h dialysis against 0.9% saline in Spectrapor tubing (Roth, Karlsruhe, FRG; cutoff 12–14 kDa) and contaminating Con A was blocked by adjusting the supernatant to 100 mM methyl-α-D-mannopyranoside.

2.4 Cytolytic assay and calculations

After 9 days of *in vitro* incubation, the lytic activity of individual cultures was determined on 10^3 ^{51}Cr-labeled (2–3 cpm/cell) target cells in a conventional 4-h ^{51}Cr-release assay [5]. Target cells were ERA-infected MC57G fibrosarcoma cells and noninfected controls or, in the case of the alloresponse, DBA/2-derived P815 mastocytoma cells. Low controls were obtained by incubation of targets with cultures (36 replicates) containing stimulator or filler cells only, resulting in a release of 10–14% of total incorporated radioactivity. A culture was considered "responding" if the release exceeded the 99% tolerance limit of the low control normal distribution (but at least 5% specific lysis) and as "antigen-specific" if it was scored as negative on the control target. The proportion of nonresponding cultures was plotted on a logarithmic scale on the y axis against the cell input on a linear scale on the x axis. The best-fitting straight line through the origin was calculated by the least square method and goodness of fit tested by a χ^2 test. Only if the single hit hypothesis could be accepted with a probability of $p > 0.05$ (range $0.25 < p < 0.9$ in reported experiments) the relative CTL-P frequency was estimated according to the Poisson statistics. All calculations were carried out on a Commodore CBM 3032 computer using software mathematically based on Lefkovits and Waldmann [6].

3 Results

3.1 Direct CTL activity measurable after *in vivo* priming

To induce a lethal infection in C57BL/6 mice, an appropriate dose (10^7 PFU) of an attenuated but still virulent strain of rabies virus ERA was applied locally by injection into the hind footpad, causing death within 6–12 days post infection. On day 4 post infection the primary immune response in the popliteal LN draining the infected area was analyzed. This infection route is closer to the natural route than the nonlethal i.p. application. Early detection time was necessary to analyze the system in a state of activation and avoid interference by the onset of acute sickness. This 4-day *in vivo* local sensitization protocol should provide information on the cytolytic activity generated *in vivo*.

Despite the evident immune reaction visible by an increase in the overall cell number from 5×10^5 in the popliteal LN of an unprimed host to 4×10^6 after antigen encounter, no significant rabies-specific cytotoxicity was detectable in the direct 4-h cell-mediated lysis assay. This failure however is not unique to rabies infection, but can also be observed when a similar protocol is applied to study the *in vivo* reaction of C57BL/6 popliteal LN cells towards a local DBA/2 lymphoid cell allograft, elicited by injecting 5×10^6 mitomycin C-treated SC into the hind footpad. The definite but rather low response to alloantigen (18 ± 2% specific lysis at an effector/target ratio of 40 : 1) is in line with earlier reports [7] and has been explained as the consequence of an *in vivo* operating type of suppressor cell contraacting the clonal expansion of sensitized pre-killer T cells [7, 8].

3.2 Detection of CTL by limiting dilution techniques

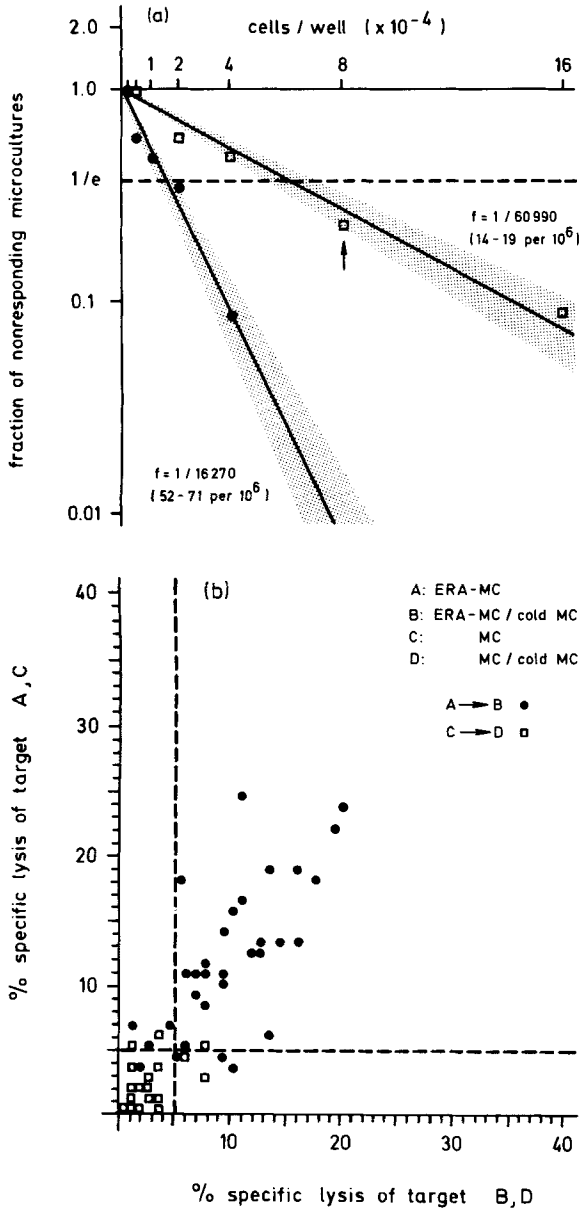
Cells of the CTL lineage may participate at different maturation stages in the acute primary immune response *in vivo*. To perform quantitative analysis we used three types of limiting

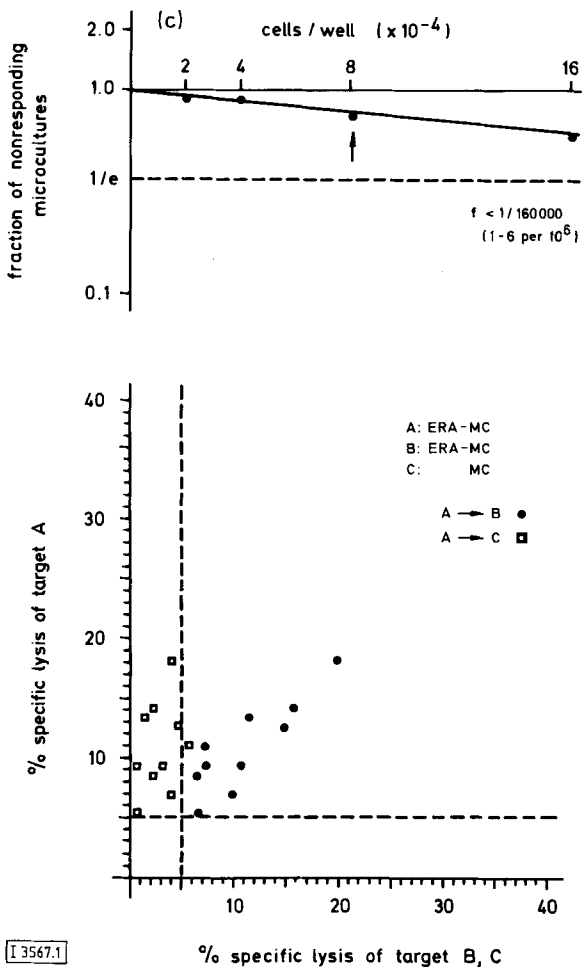
Table 1. Comparison of anti-MHC (H-2^d) with anti-ERA CTL-P/IL-CTL-P frequencies in C57BL/6 mouse popliteal LN cells[a]

Protocol	Antigen presentation		Estimate of the relative frequency (95% confidence limits)	Estimate of the absolute frequency/LN
	In vivo	*In vitro*		
1	Unprimed	MHC-antigen + IL	1/ 179 (4800–6400)[b]	2400 – 3200
2	MHC-antigen	MHC-antigen + IL	1/ 197 (3800–6400)	15000 –26000
3	MHC-antigen	IL	1/ 290 (2700–4200)	11000 –17000
1	Unprimed	ERA + IL	1/277000 (1– 6)	0.5– 3
2	ERA	ERA + IL	1/ 16270 (52– 71)	208 – 284
3	ERA	IL	1/ 60990 (14– 19)	56 – 76

a) Four to 6-week-old male (C57BL/6 (H-2^b) mice were primed by footpad injection of either 5×10^6 mitomycin C-treated DBA/2 (H-2^d) SC or 10^7 PFU of purified tissue culture (BHK)-grown ERA-strain of rabies virus. Four days later the cells of the draining popliteal LN were suspended in IL-containing medium and seeded under limiting dilution conditions in microtiter plates. After a 9-day culture in the presence or absence of antigen, the cytolytic activity of individual cultures was determined in a ^{51}Cr release assay. Target cells were either ERA-infected MC57G fibrosarcoma cells and noninfected controls for virus-specific CTL or DBA/2 derived P815 mastocytoma cells to test the alloreactive CTL. The frequency calculations were performed as described in Sect. 2.4. The estimate of the absolute frequency was calculated by correcting the relative frequency for the mean number of cells per LN: 5×10^5 (measured for a pool of 20 LN) in unprimed animals and 4×10^6 (pool of 8 LN) in alloantigen-primed or ERA-primed animals.

b) Per 10^6.





dilution protocols. The first protocol is identical with the conventional limiting dilution assay widely used to determine the frequencies of CTL-P that represent the primary repertoire in the lymphoid organs of a noncommitted animal [6, 9]. To determine these frequencies popliteal LN cells of unprimed C57BL/6 mice were cultured for 9 days under limiting dilution conditions in the continuous presence of antigen-presenting stimulator cells (allogeneic DBA/2 SC or rabies-infected MC57G fibrosarcoma cells). To ensure that the CTL-P represented the only limiting cell type, external help was provided by IL 2-containing medium [10–12]. The response of C57BL/6 popliteal LN cells to allogeneic DBA/2 SC was used as control to monitor the quality of the *in vitro* technique applied and permit comparison with published data. The results obtained are given in Table 1. In several repeated experiments the frequency of alloantigen-reactive CTL-P varied between 1/100–1/1000 cells. This is in line with experiments carried out by others [12–15] and shows the extreme deviation from the frequency of rabies-specific CTL-P (0.5–3 precursor cells/total popliteal LN) which was determined in several independent experiments (see Fig. 1c).

Figure 1. (a) Data already contained in Table 1 are depicted. Popliteal LN cells from C57BL/6 mice infected 4 days earlier with ERA rabies virus were plated in 36 replicates per concentration under restimulation (protocol 2; ●—●) or expansion (protocol 3; □—□) conditions. The mode of calculation is explained in Sect. 2.4. The shaded areas represent the 95% confidence intervals for the slopes of the regression lines.

(b) To show the specificity of CTL obtained after *in vivo* priming followed by *in vitro* IL-mediated expansion (protocol 3), one experimental point of the limiting dilution analysis (indicated by the arrow in Fig. 1a) is resolved to greater detail. The four-fold split cultures were assayed on 10^3 ERA-infected target cells and noninfected controls in presence or absence of 4×10^3 nonlabeled noninfected competitor cells. Lytic activity on targets in the presence of competitor cells is depicted on the x axis and lytic activity in absence of competitor cells, on the y axis. The dashed lines represent the tolerance limits of the low controls. Specific lysis was seen only on ERA-infected target cells, independent of the presence or absence of noninfected competitor cells (comparison A → B; ●). Activity on noninfected target cells was negligible (comparison C → D; □). It was found in separate experiments that after addition of 4×10^3 competitor cells carrying the viral antigens to responding cultures no cytolytic activity on labeled infected target cells was seen (data not shown).

(c) Frequency and specificity analysis of the primary *in vitro*-activated anti-ERA CTL (compare Table 1, protocol 1). Specificity of effector cells from one responder concentration (8×10^4/well, 36 replicates, arrow in upper part of the figure) is demonstrated in the correlation plot. The split control (comparison A → B; ●) confirms that a culture scored as positive on the infected target cells remained positive in a second determination. The same cultures were scored as negative on the noninfected target cells (comparison A → C; □). For the sake of clarity, double negative cultures are not included.

The second protocol combines the short-term (4-day) *in vivo* local antigen presentation described above with the *in vitro* technique of the first protocol. Thus, *in vivo* antigen-sensitized and pre-expanded cells were subjected to further IL-mediated expansion *in vitro*. In addition, restimulation by exposure to antigen *in vitro* may activate those precursors that have not yet acquired sensitivity to IL, as well as those *in vivo* generated mature CTL that may have already lost this reactivity. For these reasons, we expect to find the maximal frequencies following this protocol. Results are depicted in Table 1 and in Fig. 1a. In the alloresponse, the relative frequency remains constant after priming, but taking into account the change in the overall cell number a ten-fold increase in the absolute frequency was found. In the rabies system this increase is hundred-fold and the corresponding relative frequency of 52–71/10^6 cells is similar to that found after myxo- and paramyxovirus infection of mice ([16, 17]; B. Askonas, personal communication). However, there is one important difference between frequency estimates during acute virus infection and those after recovery from disease: published experiments on antiviral CTL-P frequencies were usually carried out by reactivating memory cells following long-term priming; however, in our short-term priming protocol, we assumed the antigen-specific sensitized cells were in a state of activation. Consequently, a similar frequency should be obtained by just expanding these cells in the presence of IL devoid of additional antigen.

The third protocol provides an approach to determine the frequencies of IL-sensitive CTL-P that reflect acute activation during an immune response *in vivo*. The experimental procedure was based on the widely-accepted concept that antigenic triggering induces differentiation of IL 2-insensitive CTL-P to IL-CTL-P which can be further propagated solely by the action of IL [11, 18]. This concept, valid so far under *in vitro* conditions, is now strongly confirmed by our observation that acquisition of IL responsiveness *in vivo* and propagation *in vitro* can be separated (Table 1 and Fig. 1a). The frequency of *in vivo* activated alloantigen-specific IL-CTL-P found after IL-mediated expansion is not significantly different than the frequency obtained after additional antigen restimulation. Therefore it was concluded that most of the alloantigen-activated CTL-P mature to IL-CTL-P *in vivo*, whereas most of the *in vivo* ERA-sensitized CTL-P fail to mature to detectable IL-CTL-P *in vivo* but generate an active effector progeny after additional antigen exposure *in vitro* (Fig. 1a). The cytolytic activity is specific for virus-infected cells (Fig. 1b).

An explanation for the observed difference could be that contaminating antigen may provide variable restimulation conditions. However, ERA virus does not infect resting or activated lymphoid cells (unpublished observations) and neither viral antigens [19] nor cells carrying graft alloantigen [7] are detectable in the draining LN 3–4 days after priming.

4 Discussion

It has been shown that rabies-specific CTL-P present in low numbers in the noncommitted host become sensitized and considerably expand *in vivo*, even in the course of a lethal infection. Thus, cell-mediated immunity is part of the antiviral defense mechanism, but evidently does not provide successful protection. This may be due to the low absolute numbers of participating cells compared with the reaction against foreign H-2 antigens. In addition, our data suggest that the *in vivo* process of maturation is essentially different in the two types of antigens investigated. Alloantigen- and rabies-specific IL-CTL-P may have acquired different IL sensitivity profiles at a given time after priming. A delay in the maturation of rabies-specific CTL seems reasonable considering the fact that alloantigen is already presented in association with cell surfaces, whereas free live virus has to achieve *in vivo* the appropriate presentation necessary for H-2-restricted recognition [20]. The extent of presentation may also vary in rabies strains of different virulence depending on their ability to proliferate at the site of infection. The immunosuppressive effect described for lethal infections with street rabies virus [21] may, in addition, inhibit the further *in vivo* maturation of sensitized CTL-P. To investigate these possibilities in detail and define whether the alloantigen or the rabies virus type of *in vivo* CTL-P activation reflects the normal pathway, other viruses, in particular rabies strains of different virulence, need to be tested.

Our results have a general implication in addition to their virological aspects. It was found that the majority of *in vivo* antigen-triggered and considerably expanded antiviral CTL-P cannot utilize IL 2. The generation of functionally active progeny is seen only in presence of antigen *in vitro*. One important question is whether these CTL-P themselves need further direct contact with cells carrying antigen or whether regulatory mechanisms are activated, resulting in direct or soluble helper effects that cannot be provided by IL 2 of xenogeneic origin. The inability of CTL-P to utilize the nonspecific help provided by IL 2 has been reported by Plate [22]. She describes that during early stages of syngeneic H-2-restricted (but not allogeneic) responses the CTL-P need additional MHC-restricted help provided by Ia^+ T cells in the *in vitro* system. Our data are compatible with the explanation that during the early stages of activation the majority of CTL-P require MHC-restricted soluble help that is not provided in the absence of antigen. Further analysis is required to explore this possibility.

The increase in frequency observed after local priming indicates a considerable expansion of CTL-P even during a primary response. Thus the postulated suppressive mechanism mentioned above [7, 8] may limit, but evidently does not prevent, *in vivo* proliferation. According to Teh et al. [14] a minimum clone size of 40 CTL is necessary to detect significant lysis on 300 target cells. Based on our estimate of about 1/300 alloantigen-specific *in vivo* generated IL-CTL-P (Table 1) we expected the detection threshold of the direct cell-mediated lysis assay to be at an effector/target ratio of $40 \times 300 : 300 = 40 : 1$. In fact, significant lysis was observed at this ratio and there is no need to postulate suppressive mechanisms to explain a low direct activity. The corresponding estimate for ERA-specific direct cytotoxicity predicts the threshold will be at an effector/target ratio of $40 \times 60\,000 : 300 = 8000 : 1$ and explains why CTL are not detectable in a direct 4-h assay. The operationally defined IL-CTL-P comprise IL-reactive cells of the CTL lineage, regardless of their capacity for effector function. Based on our calculations, we do not exclude the possibility that we are dealing with *in vivo* generated mature CTL rather than with CTL-P. In any case, we feel that the frequencies measured following protocol 3 represent the closest available approximation to the frequencies of CTL present *in vivo*.

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Short Papers

Kerry T. Holmes, Alan W. Hampson[+], Robert L. Raison[○], Robert G. Webster[△], William J. O'Sullivan and Carolyn E. Mountford

Commonwealth Serum Laboratories, Parkville, Victoria[+], Immunology Unit, Department of Bacteriology, University of Sydney[○] and St. Jude's Children's Hospital, Memphis[△]

A comparison of two anti-neuraminidase monoclonal antibodies by complement activation*

The specificities of two anti-neuraminidase monoclonal antibodies have been compared by their ability to fix complement. They were found to differ to some extent in their reactivity with a range of N2 influenza viruses. Thus, as in the case of anti-hemagglutinin antibodies, anti-neuraminidase monoclonal antibodies are able to detect subtle structural changes in the viral antigen. Although both monoclonal antibodies fixed complement with intact virus, neither one fixed complement when complexed with isolated neuraminidase "heads".

1 Introduction

The type A viruses of influenza are characterized by their ability to alter the antigenic character of their two surface glycoproteins, hemagglutinin and neuraminidase (Nase) [1].

Two kinds of antigenic variations occur; antigenic drift, which involves gradual changes in surface antigens, and antigenic shift, which is a sudden and complete change in one or both of the surface antigens resulting in the emergence of a new virus. Although antigenic drift occurs randomly, it is thought that the presence of antibody in an immune population selects viruses with altered antigenic determinants which have an advantage in the presence of antibody. The means by which anti-hemagglutinin antibodies neutralize the virus appears to be prevention of the virion binding to the host cell [2].

Anti-Nase antibodies are less effective than anti-hemagglutinin antibodies in neutralizing the virus *in vitro* [3] and normal titers *in vivo* do not confer complete protection against infection [4, 5]. High titers of this antibody have been found, however, in patients witch immunity to influenza [6].

The Nase of human influenza has changed dramatically only once since influenza viruses have been studied. This was in 1957 when the H2N2 strain appeared, replacing viruses with the N1 Nase. There is evidence that this enzyme is needed for both initial viral penetration [2] and for the release of newly

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Correspondence: Carolyn E. Mountford, School of Biochemistry, University of N. S. W., P. O. Box 1, Kensington, N. S. W. 2033, Australia

Abbreviations: **C:** Complement **HAU:** Hemagglutination units **Nase:** Neuraminidase **RANA:** Raison anti-Nase antibody **RIA:** Radioimmunoassay **WANA:** Webster anti-Nase antibody

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