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An immunodominant CD4+ T cell site on the nucleocapsid protein of murine coronavirus contributes to protection against encephalomyelitis

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The murine coronavirus neurotropic strain JHM (MHV-JHM) nucleocapsid (N) protein induces a strong T-helper cell response in Lewis rats. It has been shown previously that N-specific CD4+ T cells can confer protection against acute disease upon transfer to otherwise lethally infected rats. To define the major antigenic regions that elicit this T cell response, truncated fragments of N protein were expressed from a bacterial expression vector and employed as T cell antigens. Lymphocytes from either MHV-JHM-infected or immunized rats were stimulated in culture with virus antigen, grown and tested for their specificity to the N protein fragments. The carboxy-terminally located C4-N fragment (95 amino acids) induced the most pronounced proliferative response irrespective of whether the lymphocyte culture was derived from immunized or MHV-JHM-infected rats. We established T cell lines specific for the truncated N protein fragments and tested their potential to mediate protection by transfer experiments. Only the T cell line C4-N and the T cell line specific for the full-length N protein were protective. By contrast, all truncated N protein fragments elicited a humoral immune response and contained antigenic sites recognized by antibodies from diseased rats.

Introduction

Coronaviruses are responsible for a variety of important diseases in their natural hosts (Wege *et al.*, 1982). The different forms of encephalomyelitis induced by murine coronaviruses (MHV) in rodents have received much attention as a model for investigating possible mechanisms of virus-induced demyelination and multiple sclerosis in humans. The neurotropic strain JHM (MHV-JHM) causes acute and chronic demyelinating diseases in mice and rats (Cheever *et al.*, 1949; Nagashima *et al.* 1978, 1979; Sorensen *et al.*, 1980; Stohlman & Weiner, 1981; Wege *et al.*, 1984*b*; Kyuwa & Stohlman, 1990). Apart from host factors such as age and genetic background, biological properties of the virus variant also determine the outcome of infection. It was shown that the development of demyelinating lesions involves both host-specific and virus-specific immune responses (Watanabe *et al.*, 1983, 1987; Perlman *et al.*, 1987; Wang *et al.*, 1990; Zimprich *et al.*, 1991). A chronic disease state can be the consequence of incomplete eradication of the virus, following host survival of the acute stage of the infection. Therefore, interest is focused on the structure and function of viral proteins and their interactions with the immune system during the course of infection.

Studies on coronavirus infections revealed that the CD4+ T cell response is also of prime importance in this system. However, the relative contribution of cellular and humoral immunity differs according to the virus–host system under consideration. Previous investigations have shown a strong CD4+ T cell response specific for the nucleocapsid (N) protein of MHV-JHM in immunized or diseased Lewis rats (Körner *et al.*, 1991). CD4+ T cell lines with specificity for N protein have been established and transferred into Lewis rats infected with MHV-JHM and shown to confer protection.

The following studies were performed to identify the regions of the N protein that are immunodominant and important for establishing protective immunity. The N protein gene cDNA of MHV-JHM was digested with different sets of restriction enzymes. The resulting fragments were subcloned in the bacterial vector pROS and were expressed as shortened proteins. The resulting N protein fractions were employed to investigate the specific T cell response elicited by MHV-JHM in Lewis rats.

Furthermore, T cell lines were established from rats immunized with each of the truncated N proteins. Our data clearly show that the antigen C4-N (bp 1164 to 1691, coding for 95 amino acids) is a very immuno-

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dominant site for T cell proliferation and contributes to the protective immune response.

Methods

Animals. Specific pathogen-free Lewis rats (MHC RT1[l]) were purchased from the Zentralinstitut für Versuchstierzucht, Hannover, Germany. Animals were kept in a barrier system under a slightly negative pressure, a 12 h day (artificial light), and were fed and watered ad libitum. The room temperature (22 °C ± 2 °C) and the humidity (50% ± 5%) were regulated through air conditioning.

*Virus and virus antigen.*The MHV-JHM wild-type virus passage MP2S1D (Körner *et al.*, 1991) was used for infection. To stimulate T cells *in vitro* with virus antigen, a cloned Sac(−) cell culture-adapted MHV-JHM strain was propagated in suspension cultures of Sac(−) cells, purified by sucrose gradient centrifugation and then inactivated by u.v.-irradiation (Wege *et al.*, 1979, 1988).

*Bacterial strains and plasmids.*The construction of expression vectors for the N protein and truncated N protein fragments was performed using the pROS system (Bröker, 1986). In this system, the cloned genes are fused with a truncated bacterial β-galactosidase. The plasmids were used to transform competent *Escherichia coli* BMH 71-18 cells for the expression of fusion proteins (Messing *et al.*, 1977). The cDNA of the MHV-JHM protein was provided by Professor S. Siddell (Institute of Virology, Würzberg, Germany) in the form of the plasmid pTR31 (Raabe, 1987). The cDNA was cut out with *Pst*I, digested by *Fok*I and the termini were then blunt ended by employing the Klenow fragment. For ligation, the vector pROS was linearized by digestion with *Eco*RV and the resulting constructs were used for transformation. Recombinant clones were identified by restriction analysis and sequencing. The bacteria harbouring the recombinant expression plasmids were grown in LB medium containing ampicillin (100 μg/ml) at 37 °C and expression was induced by the addition of isopropyl-β-D-thiogalactoside (1 mM) when an optical density at 600 nm of 1·0 was reached. The culture was continued for 3 to 5 h.

To obtain the full-length N protein without the β-galactosidase part for immunizations, we purified the protein from *E. coli* RR1, transformed with the expression vector pEVvrf-2 containing the N gene (Crowl *et al.*, 1985; Körner *et al.*, 1991).

Preparation of antigens from N protein and truncated N protein. Following incubation, the bacteria were centrifuged, resuspended and lysed by sonication as described by Körner *et al.* (1991). Purified proteins were obtained by preparative SDS–PAGE, then eluted and the purity of the antigen was checked by immunostaining of Western blots using monoclonal antibodies (MAbs) raised against N protein (Wege *et al.*, 1984*a*). The proteins were transferred onto nitrocellulose (NC; 0·45 μm, Schleicher & Schüll) and the amount of transferred protein was estimated by amido black staining.

For T cell stimulation, purified antigen was processed according to Körner *et al.* (1991). Briefly, NC sections (5 mm to 100 mm) with bound protein were dissolved in DMSO and precipitated by bicarbonate buffer. The precipitate was collected and washed twice by centrifugation (10 min at 3000 ***g***, diluted in 3 ml of RPMI 1640 medium (Biochrom) containing 1% normal rat serum, and stored in aliquots at −20 °C.

Infection. Suckling rats (8 to 10 days old) were infected with 600 p.f.u. of MHV-JHM virus MP2S1D (30 μl) by inoculation into the left brain hemisphere.

Immunization. Eight-week-old Lewis rats were immunized by subcutaneous injection of the antigen suspension (100 μl). The antigens (proteins electro-eluted after SDS–PAGE) were emulsified in complete adjuvants (ABM) at a concentration of 8 mg/ml. To immunize rats with a vaccinia virus N protein recombinant (Flory *et al.*, 1993), the rats received two intraperitoneal (i.p.) injections (1×10^7 p.f.u. each) within 5 days. To establish T cell lines, the rats were anaesthetized and dissected 10 days after the final injection.

Serological assays, Western blots and immunostaining. Antiviral IgG was measured by ELISA on microplates coated with virus antigen (Wege *et al.*, 1984*a*, *b*). For Western blots, the virus proteins (approximately 35 μg) were separated on preparative 15% discontinuous SDS–polyacrylamide gels (Wege *et al.*, 1984*a*). To monitor the expression of bacterially expressed N protein, cell lysates were separated on 10% SDS–polyacrylamide gels. The separated proteins were electroblotted with 25 mM-sodium dihydrogen phosphate buffer pH 9·0 onto NC by electrophoresis (Wege *et al.*, 1988). The transfer was performed in a cold room at 25 V and 100 mA overnight. Filters were cut in sections and stained after blocking with Tris–HCl buffer containing 10% horse serum. The first antibody was either serum from rats or mouse MAbs raised against MHV-JHM proteins. Either peroxidase-conjugated rabbit anti-rat IgG (Dako P162) or rabbit anti-mouse IgG (Dako P161) served as a second antibody. Filters were then developed with the substrate α-chloronaphthol.

Establishment of antigen-specific T cell lines. The procedures employed were described in detail by Sedgwick *et al.* (1989) and Körner *et al.* (1991). Lymph nodes of immunized rats or spleens of infected animals were teased into a single-cell suspension and cultured in RPMI 1640 medium (Biochrom) with 1% rat serum. The bulk cultures (5×10^6 cells/ml) were stimulated with virus antigen or NC-bound N protein antigens. Within 2 to 3 days, blast cells were harvested by centrifugation through a BSA gradient and grown in RPMI 1640 medium containing interleukin 2 (IL2) and 10% fetal calf serum for 5 to 8 days. The T cell lines were then maintained by alternating cycles of restimulation with antigen as described above, in the presence of irradiated syngeneic antigen-presenting cells for 2 days, followed by growth on IL2-containing medium.

T cell proliferation assay. The assays were performed on microplates as described previously (Watanabe *et al.*, 1983; Körner *et al.* 1991). Primary T cell cultures were stimulated for 2 days with virus antigen and then grown for 5 days before the assay was performed. For each well 5×10^4 T cells, 1×10^6 antigen-presenting cells and antigen at pretested concentrations were combined in a final volume of 200 μl restimulation medium. The rate of proliferation was determined by [^{3}H]thymidine incorporation. The microparticulate antigen solution was added in different dilutions to the proliferation assays (NC at 100 and 10 μl/ml). As a control, a NC solution without specifically bound antigen was tested in parallel.

Results

Construction and expression of truncated N protein fragments

To obtain truncated N protein fragments for defining immunodominant T cell sites, the complete cDNA of the N protein was digested with sets of restriction enzymes to yield the fragments which are schematically shown in Fig. 1. A0-N represents the near full-length N protein [1450 bp, 451 N-specific amino acids (aa)]. A1-N comprises 420 bp (140 aa) from the 5′ end, A2-N the adjacent 222 bp (74 aa), followed by the overlapping C3-N (534 bp, 178 aa) and the 3′-terminal C4-N (528 bp)

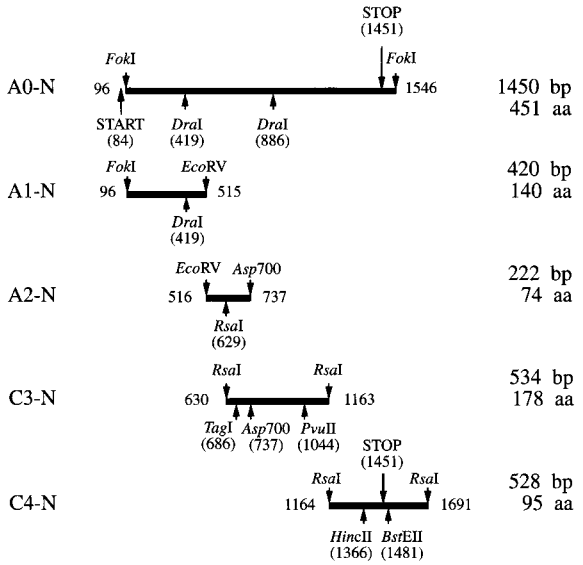

Fig. 1. Diagram of truncated N genes expressed in pROS as fusion proteins with β-galactosidase. The restriction sites employed for cloning and identification are indicated. The length of each fragment in bp and the number of N-specific amino acids (aa) are shown on the right. The truncated proteins comprise the following aa positions: A0-N 5 to 458; A1-N 5 to 144; A2-N 145 to 218; C3-N 183 to 360; C4-N 361 to 458.

which contains the stop codon and is expressed as a protein comprising 95 amino acids. Each fragment was subcloned and expressed from pROS after transformation of *E. coli* BMH 71-18. The correct insertion of the cDNA fragments was confirmed by restriction and sequence analysis. Furthermore, the level of expression and the antigen specificity was monitored by immunostaining of Western blots from bacterial lysates with a panel of N protein-specific MAbs. For each truncated N protein at least one antibody was found that recognized a specific B cell epitope (data not shown). Because the truncated N proteins were expressed as fusion products with β-galactosidase, the polyclonal T cell lines from immunized animals were specific for both truncated N proteins and the β-galactosidase part.

Specificity of virus- and N-specific CD4⁺ T cell lines for N protein fragments

In a previous study, we established a series of CD4⁺ T cell lines from MHV-JHM-infected and disease rats which were designated Vir A, B and C, respectively (Körner *et al.*, 1991). Furthermore, two cell lines were established from rats immunized with virus antigen. All these virus antigen-specific cell lines responded with strong proliferation upon stimulation with N protein antigen. In addition, we established N-specific CD4⁺ T cell lines from rats which were immunized either with bacterially expressed N protein (line N 1) or vaccinated with a vaccinia virus recombinant containing the N gene of MHV-JHM (lines Vac N 1 and Vac N 2). The N protein antigen used for immunization was expressed from the pEVvrf-2 vector without β-galactosidase as a fusion partner. The T cell lines were found to be highly specific for virus antigen as well as for the N protein (data not shown).

To obtain additional information concerning the actual site of the N protein that is responsible for T cell stimulation, we tested the panel of T cell lines established, as described above, in proliferation assays with bacterially expressed truncated N protein bound to NC. The results obtained with each type of T cell line are illustrated in Fig. 2 (*a*, *b*, *c*). Irrespective of the donor rat used for the T cell line, no significant response was elicited with the A1-N and A2-N proteins. A weak reaction to C3-N occurred with most lines. By contrast, all T cell lines tested responded strongly to the C4-N fraction. These findings suggest that at least one major antigenic site for CD4⁺ T cell-specific response is located in the carboxy-terminal region of the N protein.

Isolation of T cell lines specific for N protein subfragments

The immunogenicity of the truncated N proteins was analysed by immunization of rats and then isolation of lymphocyte lines from bulk cultures of lymphoid cells. We immunized groups of three rats with each of the truncated N proteins (A1-N, A2-N, C3-N and C4-N). T cell lines were subsequently established from each animal and tested for their antigen specificity. The lines A1-N and A2-N did not respond significantly to purified MHV-JHM antigen, but proliferated in the presence of the β-galactosidase fusion protein (Fig. 3). By contrast, the T cell lines C3-N and C4-N were also highly stimulated by virus antigen. In particular, the line C4-N always proliferated at a higher rate than the line C3-N.

Antibody response specific for N protein subfragments

The serum samples from the T cell line donors described above were tested by ELISA for antibodies raised against MHV-JHM. All rats developed a virus-specific humoral response (Table 1).

Immunostaining of Western blots by these sera confirmed the capacity of these truncated N proteins to elicit an N-specific humoral immune response (data not shown). For the Western blots, we separated MHV-JHM antigen by PAGE and transferred the proteins to NC. To obtain information on the distribution of antigenic B cell sites that have a role during MHV-JHM infection, a collection of 12 sera from Lewis rats with different disease histories was investigated by immunoblot analy-

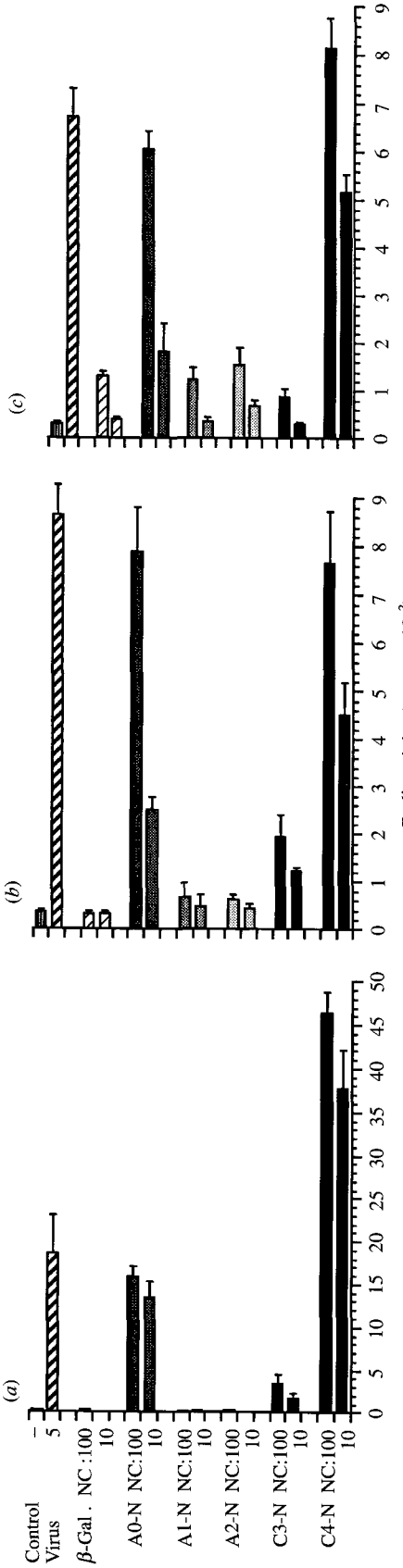

Fig. 2. Response of T cells from Lewis rats with truncated N proteins. Examples are shown for a T cell culture derived from rats with (*a*) subacute encephalomyelitis (18 days post-infection); (*b*) immunized with bacterially expressed N protein (day 11); (*c*) vaccinated with the vaccinia N recombinant virus. The primary T cell culture was stimulated with virus antigen for 2 days and grown for 5 days, before proliferation assays were performed with the antigens listed on the left side of each panel (at concentrations expressed in μg/ml for virus and μl/ml for NC solution). The columns represent the mean value of c.p.m. × 10^{-3} from triplicates.

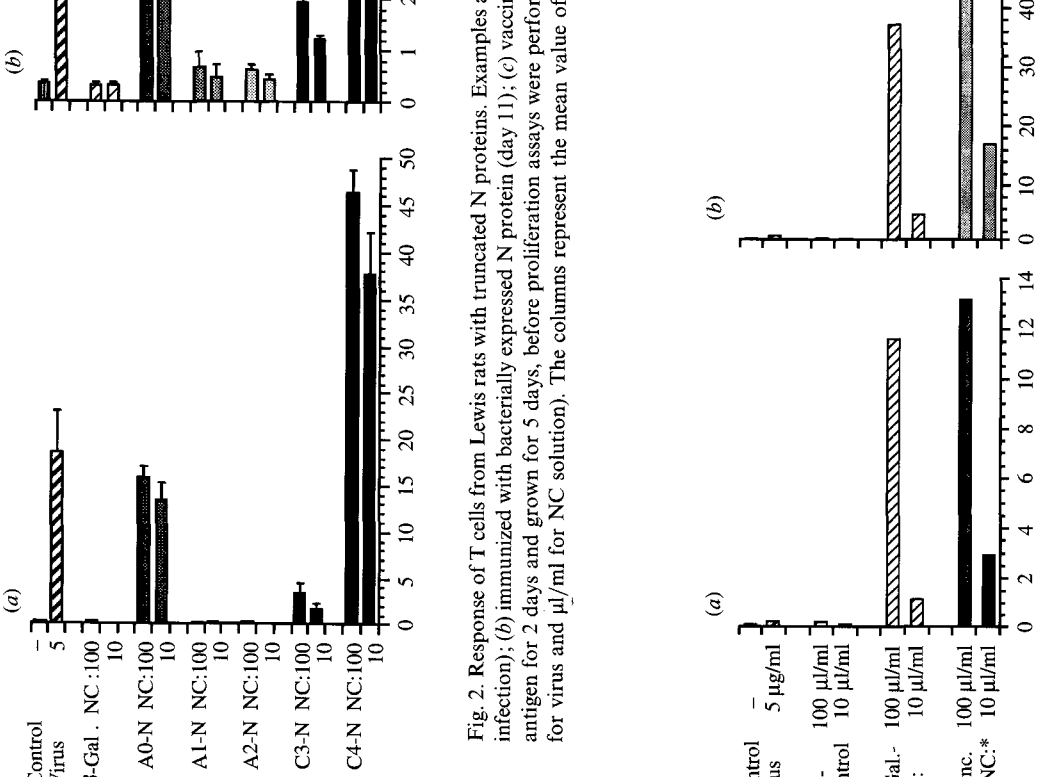

Fig. 3. Specificity of T cell lines established from rats immunized with truncated N protein fragments. The lines were tested after one cycle of stimulation. The standard deviations were for all values < 5%. * The values obtained with the following N-NC antigens are shown: (*a*) A1-N; (*b*) A2-N; (*c*) C3-N; (*d*) C4-N.

Table 1. *Antibody response (ELISA) in Lewis rats immunized with truncated N protein antigens*

Immunization with	Rat no.	Time after immunization (days)*	ELISA units†
A1-N	1	10	145
	2	11	108
	3	11	298
A2-N	4	10	580
	5	11	71
	6	10	134
C3-N	7	9	37
	8	10	23
	9	10	113
C4-N	10	8	6
	11	10	242
	12	10	56
Non-immunized	13	–	< 1
	14	–	< 1
	15	–	< 1

* The sera were taken at the same time as when the rats were dissected for preparing T cell cultures.

† The ELISA values were calculated relative to a graph obtained from experimental dilutions of a standard anti-MHV-JHM Lewis rat serum.

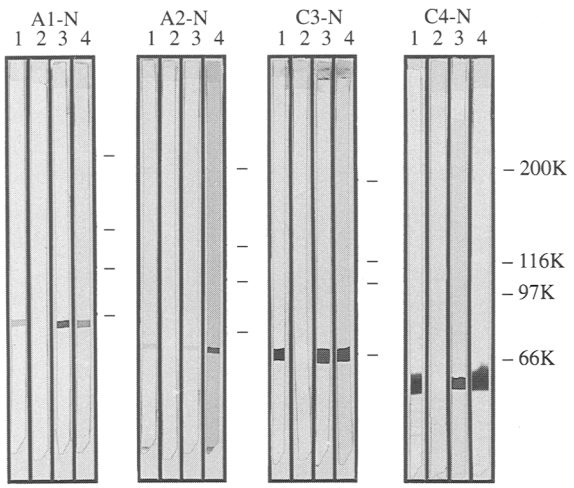

Fig. 4. Antibody specificity of sera from Lewis rats for truncated N proteins. Western blots for immunostaining were derived from 10% preparative polyacrylamide gels loaded with each antigen as indicated above the panels, in the form of bacterial lysates. Immunostaining was performed with (lanes 1) a mixture of N-specific mouse MAbs; (lanes 2) serum from a mock-infected rat; (lanes 3) serum from an MHV-JHM-infected Lewis rat 28 days post-infection,which survived subacute encephalomyelitis; (lanes 4) serum from a Lewis rat immunized with virus antigen.

sis. As shown in Fig. 4, the sera contained antibodies specific for each of the truncated N proteins. Therefore, all of the truncated N proteins contain rat-specific B cell epitopes and elicit a biologically relevant humoral immune response. Immunostaining with A1-N, C3-N and C4-N was in most cases more pronounced than for A2-N. In animals that survived subacute demyelinating encephalomyelitis a strong reaction was elicited. Infected

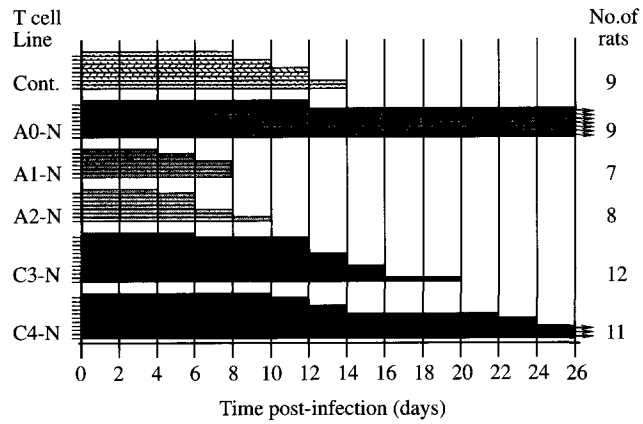

Fig. 5. Disease course after transfer of truncated N protein-specific T cells. The Lewis rats were infected at an age of 8 to 10 days intracerebrally with 600 p.f.u. of MHV-JHM. The infection was performed 1 day after intraperitoneal transfer of 5×10^6 T cells. Each animal is represented by a column indicating the survival time post-infection. The specificity of the transferred T cell line is shown on the left side of the figure, the group size is indicated on the right side. The arrows indicate rats that displayed no clinical symptoms for an observation period of at least 2 months.

rats that remained without clinical signs displayed a relatively weak response (data not shown).

Protection by transfer of N protein and N protein subfragment-specific CD4⁺ T cell lines

In order to investigate the protective capacity of T cell lines specific for truncated proteins, activated $CD4^+$ T cells (5×10^6 cells/rat) were transferred to Lewis rats by the i.p. route. The animals were challenged on the following day by intracerebral infection with a normally lethal dose (600 p.f.u., about five times the LD_{50}) of MHV-JHM. As shown in Fig. 5, control infected rats invariably died from an acute encephalomyelitis within 1 to 2 weeks. A very strong protection was induced by the T cell line A0-N. By contrast, transfer of the T cell lines A1-N, A2-N and C3-N did not result in protection. Only the T-cell lines A0-N and C4-N were capable of inducing a significant protective effect in terms of survival rate and incubation time. No clinical signs were noted in the surviving rats as observed for a period of at least 2 months.

Discussion

These studies were prompted by our earlier observation that the N protein of MHV-JHM elicits a prevailing $CD4^+$ T cell response in Lewis rats irrespective of the disease stage. The results of transfer experiments in rats challenged with an otherwise lethal dose of MHV-JHM implied that virus protein-specific $CD4^+$ T cells can mediate protection (Körner *et al.*, 1991). Here we

demonstrate that the carboxy-terminal region of the N protein is an important immunodominant antigen in eliciting a T cell response that determines the outcome of infection.

It is well known that cellular immune responses specific for internal virus proteins play a key role in a number of virus–host systems (Zurbriggen & Fujinami, 1990; Stitz *et al.*, 1990; Bankamp *et al.*, 1991). During coronavirus infection the N protein is produced in high amounts before mature virus is released. The N protein is not glycosylated and the N gene sequences are relatively conserved among different coronavirus strains (Parker & Masters, 1990). By contrast the surface proteins are glycosylated and display a high degree of genetic variability. Therefore, T cell response specific for N protein should be particularly useful in promoting virus elimination and triggering antibody response to surface proteins by the release of lymphokines. Furthermore, such a response could possibly help to control escape variants of virus with mutated surface proteins.

$CD4^+$ T cells have a propensity to help B cells regardless of their antigen specificity. It has been shown that immunization with synthetic peptides comprising an irrelevant T-helper cell epitope combined with an S protein-specific B cell epitope results in a strong protective immunity (Koolen *et al.*, 1990). Neutralizing or fusion-inhibiting antibodies elicited against viral surface protein appear rather late in infection. They are important in restricting the spread of virus and reinfection. The elimination of virus-infected targets by cell-mediated immunity may be the most important defence mechanism during the acute stage of disease. The development of a subacute demyelinating encephalomyelitis could be a consequence of insufficient virus eradication by a poor cellular response. At the chronic stage, the presence of antiviral antibodies appears to enhance the pathological response rather than protect against disease. On the other hand, the relative amount of S protein compared to N protein in brain tissue is down-regulated late in infection (Zimprich *et al.*, 1991).

The effects of $CD4^+$ T cells (helper/inducer) and $CD8^+$ T cells (CTL) on virus elimination differ to some extent with the virus–host system. To control coronavirus infections in the central nervous system of mice, both $CD4^+$ and $CD8^+$ T cells are required and act in unison (Williamson & Stohlman, 1990). Virus-specific $CD4^+$ T cells alone can prevent acute disease by a delayed-type hypersensitivity-like reaction but do not prevent replication of virus (Stohlman *et al.*, 1986). The results of transfer experiments with activated N-specific $CD4^+$ T cell lines suggest that in Lewis rats these cells alone have the capacity to eliminate virus from brain tissue (Körner *et al.*, 1991). Information obtained from other virus–host systems supports the theory that $CD4^+$ T cells can display an antigen-specific cytolytic potential. Whether this potential is also of importance during the natural course of infection remains unknown. The number of activated $CD4^+$ T cells may be insufficient to achieve virus elimination alone. We favour the concept that *in vivo* the demonstrated N-specific $CD4^+$ T cell response is an important trigger interacting with other components to eradicate infectious virus. At present no straightforward method for measuring coronavirus-specific CTL activity in the rat system is available. Some evidence for the presence of CTL in the central nervous system during recovery from acute disease was recently obtained by studies of the lymphocyte population dynamics (Dörries *et al.*, 1991).

It has been shown for a number of virus systems that N proteins can induce classical CTL reactivity. However, immunization against N protein alone is not always sufficient to provide protective immunity (Stitz *et al.*, 1990; Oehen *et al.*, 1992). Rats and mice infected with measles virus were successfully protected by immunization with N protein (Bankamp *et al.*, 1991; Giraudon *et al.*, 1991). By contrast, variable results were observed in an influenza system depending on the immunization schedules and routes of infection (Stitz *et al.*, 1990). First attempts to vaccinate Lewis rats against N protein before challenge were not successful despite their capacity to induce a specific T cell response (Flory *et al.*, 1993). Cats vaccinated against feline infectious peritonitis coronavirus with a vaccinia virus recombinant expressing N protein were not protected (Vennema *et al.*, 1991). On the other hand, the N protein of the coronavirus infectious bronchitis virus contains a T cell site that enhances protection. The antiviral protection by N protein alone might vary with the immunization protocol which influences the mode of antigen presentation or processing, the duration of T cell memory and T cell activity (Oehen *et al.*, 1992). Therefore, the passive transfer of specific T cell lines does not represent the entire reaction of the immune system during a natural infection or immunization, but is a useful tool to demonstrate T cell biological potential.

The carboxy-terminally located C4-N protein is obviously a strong antigenic determinant for T cell responses in rats. All virus-specific T cell lines reacted strongly with this antigen during proliferation. Furthermore, it was possible to grow T cell lines from animals immunized with A1-N and A2-N to produce sufficient cell numbers for transfers because the truncated N proteins were expressed from the pROS vector as a fusion protein with β-galactosidase. Our results indicate that recombinant prokaryotic or eukaryotic N proteins elicit a biologically relevant immune response.

By contrast, B cell epitopes are distributed throughout the entire length of the N protein. This conclusion is

supported by the observation that the truncated N proteins elicited a humoral response and reacted with sera from disease rats. In addition, we identified MAbs derived from mice for each of these truncated N proteins. In contrast to observations by others, we could not demonstrate a protective capacity of our N protein-specific MAbs alone, regardless of whether BALB/c mice or Lewis rats were treated.

The carboxy terminus of the N protein is rather acidic and consists predominantly of highly conserved sequences (Parker & Masters, 1990). Computer analysis of the MHV-JHM sequence for T cell motifs gave evidence that a number of possible epitopes are located at several regions within the entire N protein. Our experimental results imply that the C4-N protein represents the major antigenic region for Lewis rats. Recently, a carboxy-terminally located region of the N protein from MHV-A59 was identified in a mouse system, and was found to be important for the induction of a cytotoxic T lymphocyte response (Stohlman *et al.*, 1992). These observations support the concept that for coronaviruses also the N protein-specific response is an indispensable element in the network of defence mechanisms and complements the surface protein-specific immunity. The cellular immune response elicited by internal coronavirus proteins should be further evaluated as an important factor that regulates the outcome of infections.

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