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The anti-inflammatory effect of LS 2616 and poly I:C in coxsackievirus B3 induced murine myocarditis

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Summary

We have studied the effects of immunotherapy in coxsackievirus B3-induced myocarditis in male BALB/c mice. A single i.p. injection of the synthetic interferon inducer poly I:C conferred an almost total protection from lethality when administered at 0 h or 24 h after infection. Poly I:C treatment at 48 h after infection, as well as daily i.p. injections of the quinoline-3-carboxamide LS 2616, a new stimulator of NK-cell activity, gave no protection from lethality. The inflammatory lesions and necrosis in the ventricular myocardium 7 days after virus inoculation (3.1% of section area) were reduced in the poly I:C (24 h) treated group (1.0% of tissue section area). A less pronounced reduction was seen in the LS 2616 and poly I:C (48 h) treated groups (1.7 and 1.9% of tissue section area, respectively).

The response patterns of the studied lymphocyte subpopulations were different with these two compounds, TIB^+ (pre-B)-cells increased with poly I:C treatment (49%), but decreased with the LS 2616 treatment (65%). The Lyt 1^+ (pan T)-cells responded similarly. Poly I:C (24 h) and LS 2616 treatment tended to increase the number of class II expressing cells (1.9- and 2.9-fold, respectively). The titer of neutralizing antibodies to coxsackievirus B3 was significantly increased in the LS 2616 treated group (1:80) but not significantly so in the poly I:C treated groups (1:40) as compared to the infected and non-infected control groups (1:20 and < 1:5, respectively).

Coxsackievirus; Antiviral treatment; Lymphocyte subpopulation; Monoclonal antibody; Myocarditis

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Introduction

The enteroviruses are the causative agents of a wide spectrum of disease states, ranging from subclinical infection to fulminating multisystem infections (Woodruff, 1980). Among the enteroviruses the coxsackie B viruses are the most common agents of infective myocarditis in humans (Woodruff, 1980; Coltart et al., 1984). These cardiotropic enteroviruses replicate in the myocardial cells, which may result in subsequent development of a predominantly mononuclear cell infiltrate and necrotic lesions (Woodruff, 1980). As demonstrated in animal models, immune responses may contribute to the progression from myocarditis to dilated cardiomyopathy (Reyes and Lerner, 1976), which may eventually develop to a state where heart transplantation is the only available treatment (Johnson and Palacios, 1982).

From studies in murine models of coxsackie B virus (CBV) myocarditis there is substantial evidence that viral cytolysis is less important than T-cells and their mediators in the pathology of this disease (Woodruff, 1980; Huber et al., 1984). However, the role of natural killer cells, macrophages and antibody-dependent cell cytotoxicity (ADCC) in the defense system has not been well defined (Godeny and Gauntt, 1986). Autoimmune T-cells and autoantibodies are also formed and might partly be responsible for tissue damage (Wolfgram et al., 1985).

Consequently, our understanding of the factors that predispose to viral myocarditis and its sequelae is limited and several underlying mechanisms remain to be established. Therefore, therapy today is directed primarily toward the control of arrythmias and heart failure and presently multicentre studies are carried out to evaluate immune suppressive therapy.

In this communication we have applied a newly developed immune histochemical staining technique (Fohlman et al., 1988) to elucidate the immune cell reactions in type B3 coxsackie (CB3) virus-induced murine myocarditis after administration of two differently acting immune modulators, i.e. poly-inosinic:cytidylic acid (poly I:C) and quinoline-3 carboxamide (LS 2616). Poly I:C (synthetic double-stranded RNA) has been used as an inducer of leukocyte interferon (Tamura and Sasakawa, 1983). LS 2616 is a new immunomodulator (Stålhandske et al., 1985) and an effective stimulator of natural killer cell activity, apparently not acting via interferon production (Kalland et al., 1985).

Materials and Methods

Mice

BALB/c CUM mice were bred and maintained at the Toxicology Laboratory, Swedish National Food Administration, Uppsala, Sweden. Breeding pairs were originally obtained from Cumberland Farms, Clinton, Tennessee, U.S.A. Mice were maintained on a commercial diet (Ewos, Södertälje, Sweden) before and throughout the experiment and were housed and maintained at 23 ± 1°C. Food and water were supplied *ad libitum*. Only male mice aged 4–6 weeks were used

and their initial mean body weight (± SEM) was 20.0 ± 0.4 g. The mice were randomized into groups with similar initial mean body weight and sized to allow for losses due to lethality estimated for each group from preliminary experiments. Infected and control mice were studied simultaneously.

Virus

CB3 virus (Nancy strain) originally obtained from the late Dr. J.E. Woodruff (1970) was used. It was propagated in HeLa cells. Virus was stored at −20°C in suitable aliquots from the same stock solution until use. Concentrations of 10^7–10^8

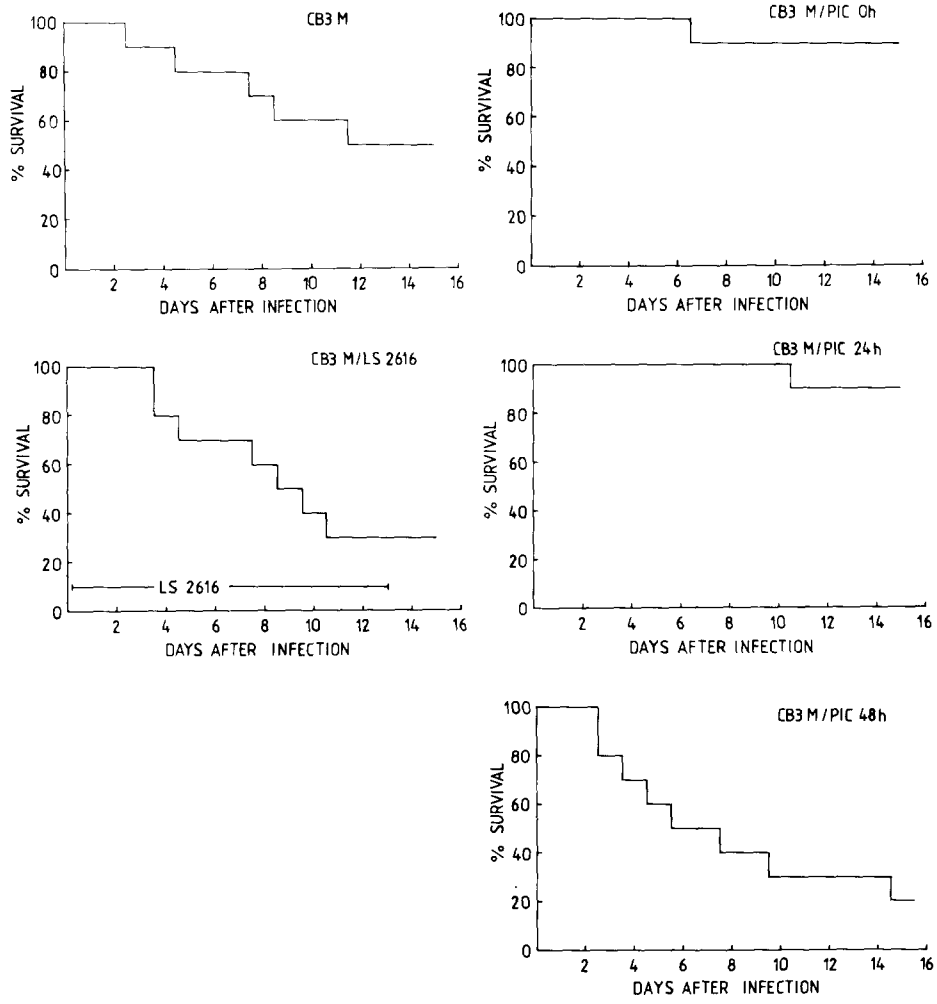

Fig. 1. Survival in male BALB/c mice infected with coxsackie virus B 3 (CB3 M) and treated with poly I:C (PIC) at various times (0, 24 or 48 h) after the inoculation, or treated daily with LS 2616 during 12 days of disease starting at the day of virus inoculation (= day 0) (n=10 in each group).

infectious particles/ml were obtained and diluted 1000-fold in order to get 5×10^4/0.5 ml, which was inoculated intraperitoneally (i.p.) into each mouse. This dose and route of administration had previously been shown to produce a median (50%) lethality at 6–8 days post inoculation in male BALB/c mice of this age and size.

Treatment protocol

After inoculation (day 0), mice were randomly divided into six groups with 10 mice in each group for lethality studies, and additional mice were similarly selected and used for conventional and immune histology. These groups were as follows: (1) non-infected and untreated control mice, (2) infected and untreated control mice, (3, 4 and 5) infected and treated with poly I:C at 0, 24 or 48 h after virus inoculation, respectively and (6) infected and treated with LS 2616 (Fig. 1). Poly I:C (Sigma, London, U.K.) was kindly provided by Prof. G. Alm (Biomedical Centre, Uppsala, Sweden) and was heat-treated according to the method of Tamura and Sasakawa (1983). It was administered as a single i.p. injection of 2 mg/kg at 0, 24 or 48 h after virus inoculation of the mice. LS 2616 (quinoline-3-carboxamide) was kindly provided by Dr. T. Stålhandske (AB Leo, Helsingborg, Sweden) and it was administered i.p. as an isotonic solution in 0.9% saline and at a dose of 80 mg/kg/day for 12 days, beginning immediately after virus inoculation. Additional non-infected mice were injected with saline, LS 2616 or poly I:C and served as treatment controls. Mice were observed and lethality was recorded daily.

Tissue preparation

On day 7 of the infection mice from each group were anesthetized using ether. The thoracic cavity was opened and the heart excised. Atria, major vessels and blood were removed and the entire remaining ventricular muscle was immediately frozen in isopentane, cooled in a dry ice/acetone mixture for immune histology. Standard formalin fixation and hematoxylin eosin staining were used for histological processing.

Pathologic examination

The ventricular myocardium was cut into 5 μm thick sections that contained ventricles and septum. Areas of myocardial necrosis, inflammatory cell infiltra-

TABLE 1

Monoclonal antibodies

Designation	Specificity	Reference
Lyt 1	most T cells	14
TIB	pre-B cells	
MAS 053	anti-Ia/antigen presenting cells	Servalab/Kemila

All monoclonal antibodies except for MAS 053 (which were obtained commercially) were harvested from cell cultures which were continuously maintained.

tion, and/or fibrosis were recorded on a grid, and the area of each of these lesions was expressed as a percentage of the total examined myocardial tissue area.

The distribution of the inflammatory lesions was determined by sequential sectioning of the entire ventricular myocardium into 5 μm sections. Every tenth section was processed by standard methods and stained with hematoxylin-eosin. The area of the inflammatory lesions on each studied level was estimated and grossly compared with that of the other tissue sections.

Immune histology

After cryostat sectioning, immune histochemical staining was carried out essentially as described by Karlsson-Parra et al. (1983). Endogenous peroxidase was blocked by incubation in 0.3% H_2O_2 for 15 min. The slides were incubated for 30 min in a humid atmosphere with 25 microliter portions of monoclonal antibody (Table 1). Biotinylated rabbit antimouse IgG was allowed to bind for 30 min. A complex of avidin DH (10 mg/ml) and biotinylated horseradish peroxidase H was then layered onto the section for 30 min. The peroxidase reaction was developed using a carbazole containing buffer for 15 min. A background nuclear stain was obtained by exposure to hematoxylin.

Stained cells showed a red "pseudomembrane" that made them easily distinguishable from non-stained cells (Fig. 2). All Lyt 1^+, TIB^+ and class II^+ cells in each studied myocardial tissue section were counted after immune specific stain-

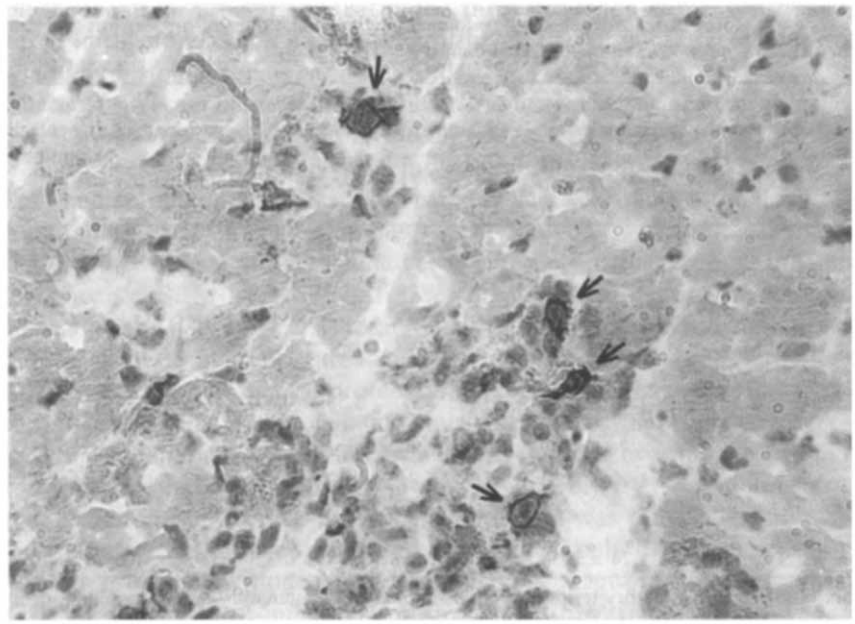

Fig. 2. A typical photograph of a myocardial tissue section in the immunohistochemical staining technique after application of the specific monoclonal antibodies against TIB (B-cell marker), Lyt 1 (now regarded as a pan T-cell marker, formerly a T-helper specificity) or MAS 053 (anti-Ia/macrophages etc).

ing. Lyt 1 is a cell surface marker involved in T-cell antigen recognition and is detectable on most T-cells. With this marker a higher percentage of cells were stained than with the other markers used (Fig. 2 and Table 2). Class II expressing cells (MAS 053-positive cells) probably represent macrophages. Each of these kinds of cells was then expressed as the number of cells per defined inflammatory area, after calculation of the total area of inflammatory lesions. Omission of the primary antibody was used as a negative control.

Neutralizing antibodies

Pooled sera from each experimental group were analyzed for neutralizing antibody titer. This was done in GMK-cells, as previously described by Torfasson et al. (1984).

Statistics

The effects of infection and anti-inflammatory treatments were calculated by means of Student's *t*-test, which compared control and experimental groups for each treatment.

TABLE 2

Effects of coxsackie virus B3 (CB3) infection and treatment with single doses of poly I:C at various times or repeated doses of LS 2616 on the development of histologic signs of myocarditis and production of neutralizing antibodies in male BALB/c mice

Treatment group	*n*	Area inflamed, % of total	Lyt 1^+/cells/ inflammatory area	TIB^+ cells/ inflammatory area	Class II expressing cells/ inflammatory area	Titer of neutralizing antibodies
Infection (CB3 virus) alone	8	3.13 ± 1.06	16.4 ± 2.9	2.13 ± 0.53	1.51 ± 0.77	1:20
Infection + poly I:C (0 h)	8	ND	ND	ND	ND	1:40
Infection + poly I:C (24 h)	8	1.03 ± 0.33	20.1 ± 3.7	3.53 ± 0.67*	4.45 ± 1.21	ND
Infection + poly I:C (48 h)	8	1.90 ± 0.63	21.5 ± 6.8	2.82 ± 0.84	1.47 ± 0.48	1:40
Infection + LS 2616	10	1.74 ± 0.36	9.7 ± 3.7	0.74 ± 0.32*	5.92 ± 3.90	1:80*

The quantitation of inflammatory lesions, specific staining of inflammatory cell subpopulations in the ventricular myocardium and determination of neutralizing antibodies were performed at 7 days after virus inoculation. Means ± SEM are given. Asterisks denote statistically significant differences ($P < 0.05$) between infected untreated and infected treated mice.

ND = Not determined.

Results

The lethality on day 7, when tissue samples were taken in this experimental model of CB3 virus-induced myocarditis in the mouse, was approximately 20% in the infected untreated control group (Fig. 1). After treatment with poly I:C at 0 h, the 7-day lethality rate was 10%; when poly I:C was given at 24 h after the infection it was 0%, and when poly I:C was administered at 48 h after the infection it was 50%. With daily LS 2616 treatment lethality on day 7 was 30%, which was not significantly different from that in the infected controls. Of the mice receiving poly I:C at 0 and 24 h post inoculation, more than 90% survived on day 16 after infection, whereas administration of poly I:C at 48 h post inoculation tended to increase the lethality rate. The pattern of lethality was similar in the poly I:C (48 h) and LS 2616-treated groups, the majority of mice dying in the early phase of the infection, corresponding to the period where the myocardial virus titer reached its peak (Reyes and Lerner, 1976). The poly I:C and LS 2616 treatment did not cause lethality of the control mice.

Histologically, on day 7 of the infection the hearts of untreated mice showed focal necrosis of myocytes, infiltration of mononuclear cells and calcification. Necrosis was observed in areas of inflammation and is therefore included in the calculated total area of myocardial inflammatory lesions. Gross inspection of serial sections from the ventricular myocardium showed no relative difference in size and number of inflammatory lesions at different section levels. Thus, the inflammatory response seems to be equally distributed throughout the entire ventricular myocardium in this infection model. None of the groups of non-infected control mice showed any histologic signs of myocarditis.

The most severe inflammatory lesions in the hearts of mice (mean =3.1% of tissue section area) were observed in the untreated CB3 virus-infected group. However, there was a considerable variability within the group (Table 2). Thus, in two of the infected mice, the myocardial histology was essentially normal with an area of inflammatory tissue corresponding to only 0.3–0.4% of the total tissue area. When these two mice were excluded from the group, the average inflammatory lesion (mean ± SEM) was 4.05 ± 1.32% and significantly more pronounced ($P < 0.05$) than in the poly I:C (24 h) treated group, which showed inflammation in 1.03 ± 0.33% of the total section area.

Apart from an almost total protection, in terms of lethality, poly I:C administration at 24 h after virus inoculation thus had a clearly suppressive effect on the myocardial inflammatory response, which was reduced by a mean of 67% of that observed in the infected untreated group. When poly I:C was administered 48 h post inoculation the average area of inflammation was reduced by 30% as compared to that of the infected untreated mice. Thus, poly I:C administration either 24 or 48 h after inoculation resulted in a smaller mean inflammatory area (Table 2). The average area of inflammation in the LS 2616-treated group was reduced by 44% of that of the infected untreated group.

Regardless of treatment, we usually observed a smaller number of class II expressing cells (probably representing macrophages) in areas of extensive myocar-

dial inflammation and destruction than in areas with low or moderate alteration. Thus, there seemed to be a negative correlation between the extent of lesions and the number of macrophages.

Although poly I:C administered 24 or 48 h after the infection resulted in a reduction of the mean total inflammatory area, the Lyt 1^+ and TIB^+-cells (pre-B cells) in the lesions were slightly increased, i.e. by 23% (NS) and 66% ($P < 0.05$) at 24 h, and by 31% (NS) and 32% at 48 h, respectively. When poly I:C was administered 24 h after the inoculation there was a 195% increase in these cells and an almost total protection from lethality. However, when poly I:C was given 48 h after inoculation the number of class II positive cells was unaffected by the treatment and no protection occurred in terms of lethality. LS 2616 also seemed to have a suppressive effect (44%) on the mean inflammatory response, but a completely different pattern of responses occurred in the lymphocyte subpopulations. Thus, the numbers of Lyt 1^+ and TIB^+-cells were both reduced, i.e. by 41% (NS), and 65% ($P < 0.05$), respectively, whereas a non-significant increase was recorded in the number of class II^+ cells (292%) that was comparable to that in the poly I:C (24 h) treated group.

The titers of neutralizing antibodies to CB3 virus 7 days after infection (Table 2) were 1:20 in the infected untreated control group and 1:40 when poly I:C was administered 0 to 48 h post inoculation. In the group treated with LS 2616 the titer of neutralizing antibodies was 1:80. There was no response (< 1:5) in the non-infected control group.

Discussion

We have applied an immune histochemical technique (Fohlman et al., 1988) to study immunotherapy in coxsackie B3 (CB3) virus-induced murine myocarditis. This infection evoked classical histologic signs of myocarditis (Woodruff, 1980). Treatment with poly I:C, administered 24 h post inoculation resulted in an almost total protection from lethality, whereas lethality tended to increase when this compound was given at 48 h post infection. LS 2616 administered daily gave no protection from lethality. The most severe inflammatory and necrotic lesions were observed in the infected and untreated control group, whereas the mean inflammatory response, as well as necrosis, was reduced in the poly I:C (24 h) treated group. A similar trend was observed in the LS 2616 and poly I:C (48 h) treated groups. The pattern of lymphocyte subpopulations in the myocardial inflammatory lesions was different with these compounds. Thus, the number of TIB^+-cells increased with poly I:C treatment, but decreased with LS 2616 treatment. With early poly I:C as well as with LS 2616 treatment, an increased mean number of class II expressing cells was recorded, as compared to that in infected controls. On the other hand, administration of poly I:C at 48 h after the infection did not affect class II cells. Both treatment regimens seemed to stimulate the production of specific neutralizing antibodies, a significant titer increase being recorded with LS 2616 (Table 2).

Preliminary data indicate that immunosuppressive therapy does not improve the

outcome of human myocardial inflammatory disease (Anderson et al., 1986). It is conceivable that a reduction of the immunological response leaves the heart less defended against the virus (Chandra and Chandra, 1986), although some data suggest that virus replication without an inflammatory response is less harmful to the host (Huber et al., 1984). Possibly immunosurveillance of virus-infected cells could be augmented and viral replication hampered by the NK cell stimulator LS 2616 and the interferon inducer poly I:C, if administered optimally.

Interferons are probably important elements in the response to several viruses exhibiting a different pathogenesis (Gresser et al., 1976). The synthetic interferon inducer, poly I:C, profoundly increases serum interferon levels and spontaneous cytotoxicity in peritoneal exudate cells of BALB/c mice (Kalland et al., 1985). Poly I:C also induces NK cell activity, stimulates phagocytosis, formation of antibodies and cell-mediated immune responses of which interferon is one of the major effector molecules (Reyes and Lerner, 1976; Abruzzo et al., 1986). However, poly I:C induced NK cell activity decreases to normal levels within 4 days after a single administration (Abruzzo et al., 1986).

Human fibroblast interferon protects against CB3 virus infection in cultures of human fetal myocytes (Kandolf and Hofschneider, 1984). Similarly, in CB3 virus infection in mice a single i.p. injection of poly I:C either within 12–48 h before or after inoculation results in a significant protection from myocarditis (Reyes and Lerner, 1976). However, if the compound is given 48 h after the CB3 virus (Reyes and Lerner, 1976) or if interferon is given therapeutically when symptoms have begun (Tyrrell, 1987) there is no protection at all. We observed similar effects related to the timing of the administration regarding both lethality and inflammatory lesions.

The interferons are efficient as immune regulatory molecules and they can affect the growth of mononuclear phagocytes either positively or negatively, depending on conditions (Russel and Pace, 1987). It has been suggested that the magnitude of the interferon production in the bone marrow dictates whether monocytopoiesis of macrophage progenitors is augmented (low concentration of interferon) or inhibited (high concentrations of interferon) (Russel and Pace, 1987). Thus, mononuclear phagocytes express class II antigens when they have been appropriately stimulated to do so. The doubled and tripled number of class II expressing cells observed with poly I:C at 48 h and LS 2616 treatment, respectively, may thus indicate rapidly mobilized phagocytic cells, ongoing reparative mechanisms and tissue protection.

NK cells influence the function of other immunocompetent cells and express their reactivity in part through the production of interferons, which in turn act as potent regulators of NK activity (Wigzell and Ramstedt, 1986). Stimulation of natural killer cells may thus have a therapeutic value and LS 2616 is an effective stimulator of NK cells, apparently not acting via interferon production (Kalland et al., 1985). Treatment with LS 2616 during the course of the parasitic disease caused by *Trypanosoma equiperdum* delayed parasitemia and was correlated with increased time of survival (Stålhandske et al., 1982). The related *Trypanosoma cruzi* may also cause myocarditis and the effector cells responsible for destruction of the parasites

are typical natural killer cells (Hatcher and Kuhn, 1982). When LS 2616 was administered to BALB/c mice during 4 days in their drinking water it increased spleen spontaneous cell-mediated cytotoxicity by 73% (Kalland et al., 1985).

Virus reaches maximum titers in myocardium at about 3 to 4 days post inoculation in the present animal model (Woodruff, 1980). According to Godeny and Gauntt (1986) there are two peaks of NK-activation in the present infection model: 3 and 7 days after the inoculation. Myocardial inflammatory lesions do not appear until day 5 and reach their peak at about day 8. This may mean that the compound LS 2616 has no real effect during the early acute part of the viremia, whereas daily treatment apparently has a protective effect on the development of myocardial inflammatory lesions (Table 2).

The number of Lyt 1^+- and TIB^+-cells varied independently of the size of the inflammatory lesion. Therefore, the number of T- and B-cells in the myocardium may be of less importance and only indicate a general presence of non-specific immune cells in the inflammatory response.

There also seems to be an adjuvant effect of LS 2616, since administration of this compound resulted in enhanced neutralizing antibody titer. Accordingly, it has been reported that LS 2616 greatly increases the number of anti-SRBC Ig-secreting cells six days after a single LS 2616 treatment (T. Stålhandske, personal communication).

Thus, LS 2616 seems to limit the inflammatory reaction in the late phase of the infection, to increase antigen presentation, to raise the number of class II expressing cells, and to stimulate production of neutralizing antibodies. Possibly disease progression towards dilated cardiomyopathy might then be halted in surviving animals. On the other hand, poly I:C seems to exert its protective effects during the early phase of the infection by preventing virus replication, cell damage and lethality. When poly I:C was administered early it also seemed to stimulate class II antigen expression (macrophages) that may be of importance for reparative responses and restoration of the heart muscle pump. No clear-cut correlation existed between the immune cells studied here and the prognosis of CB3 virus-induced myocarditis. Thus, it still remains subject of future research to define any immune function factor that correlates with the severity of the myocardial inflammatory lesions.

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