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Elucidation of mode of retroviral-inhibitory effects of imexon through use of immune competent and severe combined immune deficiency (SCID) mice

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Summary

Mice infected with various tumor retroviruses have been used as models for evaluating therapeutic substances for the treatment of some cancers, and more recently, for human immunodeficiency virus (HIV) infection, the causative agent of acquired immune deficiency syndrome (AIDS). Consequently, there is a need to determine the ability of biological response modifiers (BRMs) to specifically reduce virus-infected cells, as compared to their non-specific anti-proliferative effects. To address this need, a BRM, imexon, was evaluated in this study using three strains of mice having different Friend virus (FV)-specific immunological capabilities. The first strain, (B10.A × A/WySn)F_1, was genetically capable of producing FV-specific neutralization and cytotoxic antibodies, the second, Balb/c, was not, and the third, SCID mice, lacked functional T and B cell immunity. Imexon treatment reduced virally-induced splenomegaly in all 3 strains; however, the concentration of splenic viral infectious centers (IC) were not affected. Since imexon was efficacious in reducing splenomegaly in SCID mice, the mode of action was concluded to not require functional T or B cell immunity. The observation that imexon did not affect splenic IC titers also suggested that imexon did not specifically eliminate virally infected cells, but may have functioned by other mechanisms. This study also demonstrated the use of various mouse strains as a strategy for delineating the modes of action of BRMs against murine retroviral infections.

AIDS; HIV; Friend virus; Severe combined immune deficient (SCID) mice; Biological response modifier (BRM); Imexon

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Introduction

Mice infected with various oncogenic retroviruses have been frequently used as models for evaluating potential anti-proliferative substances. Within the last decade, a human retrovirus, human immunodeficiency virus (HIV), has been determined to be the causative agent of acquired immune deficiency syndrome (AIDS). Since HIV does not infect animals that could be routinely used for therapeutic studies, murine retroviruses have been successfully employed as models for HIV infection (Ruprecht et al., 1988; Mosier 1986; Chirigos et al., 1990; Ruprecht et al., 1990; Morrey et al., 1990a and 1991). In addition to the use of antiviral substances targeted specifically to the replication of HIV, treatment with biological response modifiers (BRMs) have been the subject of considerable research efforts as an approach for enhancing current therapies (Polsky and Armstrong, 1988). There is a need, however, to determine the ability of BRMs to specifically reduce virus-infected cells, as compared to their non-specific anti-proliferative effects.

Recent studies using the Friend virus (FV) infection (Morrey et al., 1990a; Morrey et al., 1991), showed that the putative BRM, imexon (4-imino-1,3-diazobicyclo-(3.1.0)-hexan-2-one) (Bicker, 1978), was efficacious in reducing disease parameters in FV-infected mice. Imexon also possesses anti-proliferative activity of both retroviral and non-retroviral etiology (Bicker, 1978; Bicker et al., 1978; Micksche et al., 1978; Micksche et al., 1982). It is important to determine if the mode of action of a BRM is to elicit immune responses that specifically reduce the virus or virus-infected cell (Morrey et al., 1991).

It has long been known that various strains of mice have different genetic capabilities to respond immunologically to murine retroviruses. More specifically, genetic loci have been identified that affect the production of FV-specific neutralizing and cytotoxic antibodies (Chesebro and Wehrly, 1976a; Chesebro et al., 1979; Chesebro and Wehrly, 1979; Doig and Chesebro, 1979), FV-specific cytotoxic T cell activity (Chesebro and Wehrly, 1976b; Miyazawa et al., 1988), FV-specific T cell responsiveness (Britt and Chesebro, 1983) and the animal's ability to immunologically respond early or late in the infection (Britt and Chesebro, 1983). In this study, the mouse strains used were (B10.A × A/WySn)F_1, Balb/c and severe combined immune deficiency (SCID) mice. The (B10.A × A/WySn)F_1 mice have the ability to produce (FV)-specific neutralizing and cytotoxic antibodies, but no detectable FV-specific cytotoxic T lymphocyte activity (Chesebro and Wehrly, 1976a). Balb/c mice produce no such immune responses. The SCID mice lack B and T cell immunity and have small displastic thymus in infancy and an atropic lymphoid system (reviewed in Bosma, 1989). The purpose of this study was to explore the use of these strains of mice possessing different FV-specific immune capabilities to aid in the delineation of the mode of action of imexon.

In a previous publication (Morrey et al., 1991), we described imexon treatment using an IC's assay of (B10.A × A/WySn)F_1 mice. We observed that

splenomegaly was reduced, but IC's/splenocyte was not affected, which led to the hypothesis that imexon is not specifically reducing FV-infected cells by an immune response involving T or B cells. The present study was designed to test the hypothesis by using other strains of mice that have reduced T and B cell FV-specific responsiveness. A strategy for delineating the mechanism of action of other BRMs using different strains of mice with different immune capabilities is described.

Materials and Methods

Mice

Female B10.A and male A/WySn mice (Jackson Laboratories, Bar Harbor, ME) were mated to produce the (B10.A × A/WySn)F_1 mice. These F_1 hybrid mice were supplied as young adults weighing 18–23 g by Simonsen Laboratories (Gilroy, CA). Female 14–16 g Balb/c mice were also obtained from Simonsen Laboratories.

Breeding pairs of SCID mice were obtained from Dr. Norman Klinman at Scripps Institute (La Jolla, CA). The SCID mice were housed in microisolator cages (Lab Products, Inc., Maywood, NJ) containing sterilized bedding (Pawus Bedd), food (Wayne Lab Blox), and water. The cages were maintained in HEPA-filtered horizontal laminar flow hoods or similarly filtered ventilated animal racks (Lab Products). The SCID mouse colony was managed so that the incidence of leaky phenotype was kept to a minimum of less than 5% (Bosma, 1989). Methods for determining immunoglobulin levels and subpopulations of splenocytes are described below. Animal care was authorized and in accordance with Utah State University Institutional Animal Care and Use Committee.

Virus

The Lilly-Steeves B-tropic strain of FV complex (Morrison et al., 1986) was obtained from Dr. Bruce Chesebro (NIH, NIAID, Rocky Mountain Laboratories, Hamilton, MT). The virus consists of helper Friend murine leukemia virus (F-MuLV) and defective spleen focus-forming virus (SFFV). An FV stock was prepared in mice as previously described (Morrison et al., 1986).

Test compound

Imexon was provided by Boehringer Mannheim GmbH (Indianapolis, IN) for these studies. It was stored at room temperature and made fresh in sterile saline each day before use.

Viral assays

Splenomegaly and viral infectious centers (IC's) of the spleen and plasma were used as indicators of viral infection and were described previously (Morrey et al., 1990b; Morrey et al., 1991). A focal immunoenzyme assay (FIEA) (personal communication, Dr. Bruce Chesebro, NIAID, NIH, Hamilton, MT) employing monoclonal antibody (MAb) 48 (Sitbon et al., 1985; Evans and Morrey, 1987) specific for envelope of Friend murine leukemia virus (F-MuLV) was used as an indication of productively infected cells in the spleen and to quantitate cell-free FV in the spleen and plasma. Serial dilutions of either unfractionated spleen cells or heparinized plasma were added to an 18-h monolayer of *Mus dunni* cells seeded on 24-well tissue culture plates. After cultivation for a 5-day period, confluent monolayers were incubated with MAb 48, rinsed and fixed with methanol for 5 min. After rinsing 2 times to remove methanol, the wells were incubated for 45 min at room temperature with peroxidase-conjugated goat anti-mouse immunoglobulin (Cappel, West Chester, PA). A substrate, 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO) in dimethyl formamide (4 mg/ml), was then added after rinsing 2–3 times with 0.01 M Tris, pH 7.6, 0.15 M NaCl, 0.002 M ethylenediaminetetraacetic acid and incubated in the dark for 20 min. Plates were rinsed with distilled water and foci counted and expressed as IC/10^6 splenocytes or focus-forming units (FFU)/ml.

Northern dot blot hybridization

Quantitation of viral RNA as performed by northern dot blot hybridization was previously described (Morrey et al., 1990b; Morrey et al., 1991). In the test, 5×10^6 spleen cells were lysed in a solution of vanadyl nucleoside as an RNase inhibitor, and Nonidet P-40. The nuclei were pelleted and cytoplasmic lysates were carefully removed and frozen at $-20°C$ if storage was needed. After phenol/chloroform extraction, ethanol precipitation and suspension of the RNA in buffer containing vanadyl nucleoside, the RNA was denatured in 12 × SSC and 15% formaldehyde at 60°C. The solution was blotted onto nitrocellulose (NC) paper and hybridized with DNA probe specific for F-MuLV long terminal repeat. After exposure of X-ray film to the NC paper, the intensity of the dots containing the samples were compared to serially diluted F-MuLV single-stranded RNA standards made from transcription of a molecular clone of F-MuLV-57 (Evans and Morrey, 1987) in a plasmid containing a T7 RNA transcription promoter.

Splenocyte enumeration

Anucleated splenic red blood cells were lysed with Lysing and Hemoglobin Reagent (Baxter Scientific, Sunnyvale, CA) and enumerated by a Coulter Counter Model F (Coulter Electronics, Inc., Hialeah, FL). The total number of

splenocytes per total spleen were calculated. These values were also used to calculate the IC's/10^6 splenocytes. Subpopulations of mouse splenocytes were enumerated in SCID mice with a fluorescence activated cell sorter (FACS) (EPICS-C, Coulter Corp., Hialeah, FL). Dispensed splenocytes were reacted with fluorescein isothiocyanate- or phycoerythrin-labeled anti-Thy 1.2 and anti-IgG antibodies (Beckton-Dickinson, Cockeysville, MD). The SCID mouse colony was monitored for 'leaky phenotypes' by assaying subpopulations of splenocytes (Morrey et al., 1991) and serum immunoglobulin (Radial Immunodiffusion Kit, The Binding Site, Birmingham, England).

Experimental design

Balb/c and (B10.A × A/WySn)F_1 mice were treated intraperitoneally (i.p.) once daily for 13 days beginning 1 day after initial FV inoculation with 110 mg/kg/day imexon or placebo. Viral and immunological parameters were obtained 1 day after the last treatment (day +14) and on day +35 after FV antibody formation had been shown to occur (Morrey et al., 1990b). Seven mice were used in each group.

To determine if SCID mice were susceptible to FV infection, 8 mice were inoculated with 3.8×10^4 50% infectious doses (ID50) and spleen weights, splenic IC titers and plasma FV titers were determined on days 9, 16 and 28 after viral inoculation where the numbers of mice were 3, 3 and 2, respectively.

FV-infected SCID mice were treated i.p. with 110 mg/kg/day of imexon or placebo once daily for 13 days beginning 1 day after initial infection. Nine FV-infected mice were in each group. As toxicity controls, 5 sham-infected mice in each group were either treated with imexon or placebo. Viral and immunological parameters were obtained 1 day after the last treatment on day +14. Statistical analysis was performed by one-way analysis of variance.

Results

Imexon treatment in (B10.A × A/WySn)F_1 and Balb/c mice

Disease parameters as assayed on days 14 and 35, respectively, are summarized in Tables 1 and 2. The effects of imexon treatment on viral parameters were essentially the same in both (B10.A × A/WySn)F_1 and in Balb/c mice. On day 14, the spleen weights in imexon-treated mice were markedly reduced as compared to placebo-treated mice. Nevertheless, the IC and plasma FV titers were unaffected at this time. As expected, the Balb/c mice appeared to be the more susceptible to the FV disease as indicated by greater splenomegaly. The ability of (B10.A × A/WySn)F_1 mice to produce FV-specific antibody is reflected in a reduction of viremia (plasma FV) on day 35, as compared to no reduction of viremia in Balb/c mice (Table 2).

The ability of imexon treatment to inhibit splenomegaly, as observed on day

TABLE 1

Effect of intraperitoneal imexon treatment[a] on FV disease parameters on day 14 in (B10.A × A/WySn)F_1 and Balb/c mice

Mouse strain	Dosage (mg/kg/day)	Toxicity controls		FV-infected		
		Survivors/total	Mean spleen wt. (mg ± 2SE)	Mean spleen wt.[b] (mg ± 2SE)	Splenic FV IC (mean $\log_{10}/10^6$ splenocyte ± 2SE)	FV in plasma (mean $\log_{10}$ FFU/ml ± 2SE)
(B10.A × A/WySn)F_1	110	7/7	67 ± 9	107 ± 27*	3.52 ± 0.63	3.10 ± 0.78
	Placebo	7/7	–	473 ± 177	4.01 ± 0.28	2.74 ± 0.52
	Normal	7/7	77 ± 10	–	–	–
Balb/c	110	7/7	66 ± 6	617 ± 247*	4.34 ± 0.41	2.81 ± 1.11
	Placebo	7/7	–	1337 ± 298	4.45 ± 0.33	3.56 ± 0.57
	Normal	7/7	87 ± 12	–	–	–

[a]Treatment schedule: qd × 13 beginning 1 day post-FV inoculation, i.p.
[b]Seven mice were used in each treatment group.
*$P < 0.01$, as compared to placebo controls.

TABLE 2

Effect of intraperitoneal imexon treatment[a] on FV disease parameters on day 35 in (B10.1 × A/WySn)F_1 and Balb/c mice

Mouse strain	Dosage (mg/kg/day)	Toxicity controls		FV-infected		
		Survivors/total	Mean spleen wt. (mg ± 2SE)	Mean spleen wt.[b] (mg ± 2SE)	Splenic FV IC (mean $\log_{10}/10^6$ splenocyte ± 2SE)	FV in plasma (mean $\log_{10}$ FFU/ml ± 2SE)
(B10.A × A/WySn)F_1	110	7/7	73 ± 10	829 ± 538	2.66 ± 0.58	0.97 ± 0.36
	Placebo	7/7	–	1113 ± 813	2.42 ± 1.16	0.70 ± 0.00
	Normal	7/7	71 ± 9	–	–	–
Balb/c	110	7/7	107 ± 8	1850 ± 228	4.42 ± 0.33	3.50 ± 0.28
	Placebo	7/7	–	2086 ± 121	4.13 ± 0.51	3.70 ± 0.27
	Normal	7/7	113 ± 19	–	–	–

[a]Treatment schedule: qd × 13 beginning 1 day post-FV inoculation, i.p.
[b]Seven mice were used in each treatment group.

14, was diminished by day 35 in both strains of mice (Table 2). By day 35, the imexon therapy also did not have an effect on IC or plasma FV titers.

Susceptibility of SCID mice to FV infection

The experiment to determine the susceptibility of the SCID mouse to the B-tropic strain of FV infection is summarized in Table 3. As expected for a susceptible mouse strain, spleen weights were not yet dramatically increased by

TABLE 3

Susceptibility of SCID mice to FV infection[a]

Days post-virus inoculation	Spleen weight (mg)[b]	Splenic FV IC ($log_{10}/10^6$ splenocytes)	FV in plasma (log_{10} FFU/ml)
9	90	4.5	3.7
	80	4.3	4.3
	60	4.0	3.6
16	1500	ND[c]	ND[c]
	440		
	1270		
28[d]	1960	~6	6.4

[a]i.p. inoculation of a 3.8×10^4 ID50 dose of B-tropic FV.
[b]Mean spleen weights of normal SCID mice were 60 mg.
[c]Not done.
[d]One of the two remaining mice died at day +27.

day 9, but substantial virus titers were noted in the spleens and plasma. By day 16, the mice tested had increased splenomegaly, again typical of a highly susceptible mouse strain. By day 28, one of the two infected mice had died. The remaining animal had virus with a titer of $>10^6$ in both the spleen and plasma, and the spleen weighed nearly 2 grams, verifying the high sensitivity of SCID mice to the B-tropic strain of FV.

Imexon treatment in SCID mice

The SCID mice were inoculated with FV and then treated on the same schedule and dosage of imexon used for (B10.A × A/WySn)F_1 and Balb/c mice described above. Viral parameters were determined on day 14. The spleen weights were markedly reduced in the imexon-treated mice (Table 4); therefore, the immunosuppressed status of SCID mice did not appear to affect the efficacy of imexon. The efficacy of imexon to reduce splenomegaly in SCID

TABLE 4

Effect of intraperitoneal imexon treatment[a] on FV disease parameters on day 14 in SCID mice

Dosage (mg/kg/day)	Toxicity controls		FV-infected					
	Survival/total	Mean[b] wt. change (g)	Mean spleen wt. (mg ±2SE)	Survival/total	Mean spleen wt. (mg ±2SE)	Splenic FV IC (mean $log_{10}/10^6$ splenocyte ±2SE)	FV in plasma (mean log_{10} FFU/ml ±2SE)	Mean FV RNA (ng/splenocyte ±2SE)
110	5/5	−1.5	26±10	9/9	272±78*	4.94±0.37	5.87±0.63	38.33±4.87
0	5/5	+0.1	30±6	9/9	1076±344	4.23±1.09	6.15±1.28	50.09±36.18

[a]Treatment: qd × 13 beginning 1 day post-FV inoculation.
[b]Difference between weights at start of treatment and 1 day following final treatment.
*$P < 0.01$.

mice (75% reduction) appeared to be comparable to that seen in (B10.A × A/WySn)F_1 (77%) and Balb/c mice (54%) (Tables 1 and 4).

As noted with the other two mouse strains used, imexon treatment did not have an effect on IC or plasma FV titers in SCID mice (Table 4). Additionally, viral RNA was also not significantly affected by imexon treatment (Table 4). The SCID mice were devoid of functional T and B cells (data not shown).

Discussion

We have previously shown that imexon treatment was efficacious in reducing FV-induced splenomegaly in (B10.A × A/WySn)F_1 mice (Morrey et al., 1990a; Morrey et al., 1991); these results were similar to those reported by others (Bicker 1978; Chirigos et al., 1990). In our earlier study (Morrey et al., 1991), the IC viral parameter was also used, which was considered to be an indication of specific reduction of virus infected cells. If the numbers of splenocytes, including both erythroleukemia and normal splenocytes, were non-specifically reduced due to a diminishing spleen size, then the number of virus-expressing cells per 10^6 splenocytes would probably not be significantly altered; however, if the FV-infected cells were specifically reduced, in relation to other splenocytes, one might expect this parameter to be markedly reduced. This hypothesis was supported by the observation that IC/10^6 splenocytes were greatly reduced in mice treated with azidodeoxythymidine (AZT), which is known to act directly on the replication of the virus and subsequently reducing the numbers of retrovirus-infected cells (Morrey et al., 1990b). In the imexon study cited above (Morrey et al., 1991), however, IC/10^6 splenocytes were not significantly reduced in contrast to a significant reduction in splenomegaly. This caused us to consider that the mode of action of imexon may not be specific reduction of virus-infected cells by FV-specific immune functions. To evaluate this specific question and to further develop this murine retrovirus model for the evaluation of BRMs, (B10.A × A/WySn)F_1, Balb/c and SCID mice were used, since they all possessed different FV-specific immune capabilities.

The efficacy of imexon treatment in (B10.A × A/WySn)F_1 mice, which are genetically capable of eliciting FV-specific neutralizing and cytotoxic antibodies, was comparable with results using Balb/c mice which do not possess this FV-specific immune capability. Imexon treatment was efficacious in reducing splenomegaly in both strains of mice, whereas the IC/10^6 splenocytes and plasma FV were not reduced significantly in either strain. This suggested that imexon was not employing FV-specific neutralizing or cytotoxic antibodies to reduce virus load and splenomegaly.

The observation that the effects of imexon on FV-induced splenomegaly were essentially the same in two strains of mice that have different FV-specific immune capabilities prompted us to evaluate anti-FV efficacy in a severely immunodeficient SCID mouse. The SCID mouse strain genetically lacks T and

B cell immunity, and was derived from B type mice known to be susceptible to B-tropic FV used in this study. It was not known for certain, however, that the SCID mice would be susceptible, because they might have lacked the proper target cells to initiate infection (Evans and Morrey, 1987). In this study, SCID mice inoculated with B-tropic FV developed splenomegaly with high virus titers in the spleens and plasma at levels typical of a highly susceptible mouse strain. Since no immunoglobulin was detected in the SCID mouse colony, there should be no FV-specific antibody. The very high FV plasma titers reflected the inability of SCID mice to elicit an antibody response.

If imexon required any T or B cell immunity to be efficacious for reducing FV infection, it was anticipated that the drug would not have been effective in SCID mice. Conversely, if the mode of action of imexon were as an anti-proliferative agent, it was theorized that the drug may have been effective. Despite the congenital defect of SCID mice to not mount T and B cell immunity, imexon treatment was still effective in reducing splenomegaly. However, therapy with imexon did not significantly reduce IC/10^6 splenocytes, plasma FV titers or FV RNA.

These data indicated that T and B cell immunity was not required for efficacy of imexon to reduce splenomegaly in this FV infection. The inability of imexon treatment to reduce IC splenocytes suggested that other arms of the immune system, such as NK cell activity and macrophage function, are not an important mode of action, because these immune functions would be expected to specifically eliminate FV-infected cells.

Considering these results using strains of mice possessing different retroviral-specific immune capabilities, a strategy is proposed for evaluating BRMs in murine retrovirus infections used as models for HIV infection or cancer. Various BRMs should first be evaluated in a retroviral-specific immune competent mouse to determine the BRM having the greatest efficacy. The active BRM should then be studied in a mouse strain that is greatly immunosuppressed, such as the SCID mouse. Other strains of mice possessing different aspects of retroviral-specific immunity might also be used to further delineate which arm of the immune system might be involved. We have used this strategy in the present studies to indicate that imexon, a recognized BRM, does not require functional T and B cell immunity and does not specifically eliminate virus or viral infected cells.

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