F_0 necropsy: Two females died on study or were euthanized moribund. One from group 4 was found dead on PND435. Gross examination revealed prolapsed rectum with associated necrosis. The death was determined to be result of the prolapse and not a result of natalizumab treatment. A second female from group 4 was euthanized moribund on GD163 after cesarean delivery of her fetus due to breech presentation. This death was not related to natalizumab treatment.

No effects of natalizumab treatment was noted for clinical signs, body weight or food consumption for the females.

A 5% post-partum infant loss rate was recorded. All infant deaths occurred during the first round of the study. Two infants were from control animals and showed signs of possible maternal abuse. The third infant death was from a group 4 female, found dead on PND 1. The gross examination of that infant showed an enlarged spleen and dark red thymus. The cause of death was not determined but the enlarged spleen was most likely an effect of natalizumab treatment.

F_1 physical development:

- No effects attributable to natalizumab treatment were noted for infant clinical chemistry or coagulation parameters.
- Infant hematology was affected by treatment of the pregnant mother with natalizumab. WBC increases were observed in PND 28, 56 and 84 in infants born to mothers from group 4 (natalizumab treatment from GD20 to term). The increases were 1.2 to 1.6 more than control and were attributable to increases in lymphocyte populations. Lymphocyte populations for group 4 infants were 1.2 to 2.1 more than control.
- A small but not statistically significant (except for PND 28 and 56 lymphocyte counts) decrease in EBC and lymphocyte counts was noted.
- Increased levels of nRBC was observed in group 4 infants at PND 28, 56 and 84. Increases were noted in both incidence and magnitude. Cumulative incidence for group 4 infants relative to control was 14/89 (16%) for control and 29/41 (71%) for group 4.
- Significant increases in platelet counts were noted for group 4 infants (PND28, 56, 84 and 112. Counts were within normal limits on PND 140. This effect was not noted for group 3 infants.

Table 4 Infant Hematology Parameters

Group	Day	Parameter[a]					
		WBC (10^3/µL)	Lymphocytes (10^3/µL)	Monocytes (10^3/µL)	Neutrophils (10^3/µL)	NRBC (#/100 WBC)	Platelets (10^3/µL)
1	PND28	12.7 ± 6.3	9.4 ± 5.8	0.0 ± 0.1	2.9 ± 2.3	0 ± 0	388 ± 102
	PND56	11.0 ± 3.3	8.7 ± 2.6	0.0 ± 0.1	2.1 ± 0.9	0 ± 0	448 ± 127
	PND84	13.9 ± 6.0	10.8 ± 5.3	0.0 ± 0.0	2.9 ± 1.7	0 ± 0	560 ± 174
	PND112	13.7 ± 4.3	11.0 ± 4.5	0.0 ± 0.1	2.5 ± 1.2	0 ± 1	832 ± 799
2	PND28	14.0 ± 8.3	10.3 ± 7.3	01. ± 0.2	3.1 ± 1.4	0 ± 1	448 ± 102
	PND56	11.1 ± 3.3	8.5 ± 3.1	0.0 ± 0.1	2.3 ± 1.2	0 ± 1	411 ± 125
	PND84	13.9 ± 4.3	10.8 ± 3.5	0.0 ± 0.1	2.9 ± 2.5	0 ± 0	578 ± 186
	PND112	17.6 ± 14.9	15.1 ± 13.9	0.0 ± 0.0	2.2 ± 1.1	0 ± 0	702 ± 271
3	PND28	9.3 ± 3.1	6.5 ± 3.3*	0.0 ± 0.0	2.6 ± 2.2	0 ± 1	335 ± 145
	PND56	9.5 ±2.3	7.1 ± 2.2*	0.0 ± 0.1	2.1 ± 0.6	0 ± 0	390 ± 127
	PND84	12.3 ± 3.8	9.6 ± 3.6	0.0 ± 0.1	2.6 ± 1.2	0 ± 0	551 ± 176
	PND112	11.7 ± 2.9	8.8 ± 2.9	0.0 ± 0.1	2.7 ± 1.6	1 ± 3	909 ± 879
4	PND28	16.9 ± 4.2*	12.8 ± 3.0*	0.1 ± 0.1	3.6 ± 2.6	4 ± 3*	317 ± 72*
	PND56	18.2 ± 4.4*	15.1 ± 3.4*	0.0 ± 0.1	2.7 ± 1.3	3 ± 2*	316 ± 83*
	PND84	21.5 ± 7.4*	18.1 ± 7.3*	0.0 ± 0.0	2.8 ± 0.8	1 ± 2*	417 ± 150*
	PND112	15.2 ± 7.4	12.1 ± 5.5	0.0 ± 0.0	2.8 ± 1.7	0 ± 0	487 ± 243*

[a] Mean value ± SD – for all primary WBC types regardless of statistical significance plus any additional parameters showing statistically significant changes

* Statistically significant at $p < 0.05$, ANOVA with Dunnett's t-test

F_1 immune parameters:

Serum immunoglobulin levels:

No effects of natalizumab treatment on infant serum immunoglobulin levels was noted at 6 months of age.

FACS analysis:

APPEARS THIS WAY
ON ORIGINAL

Table 6 Maternal FACS Parameters

Group	Day	Parameter[a]			
		Lymphocytes (%)	$CD3^+$ (%)	$CD20^+$ (%)	$CD16^+$ (%)
1	GD70	45 ± 13	65 ± 8	21 ± 7	11 ± 6
	GD100	46 ± 12	64 ± 8	20 ± 6	12 ± 7
	GD150	44 ± 13	65 ± 8	20 ± 6	12 ± 6
	PND28	50 ± 14	62 ± 10	22 ± 8	14 ± 9
2	GD70	42 ± 14	70 ± 9	17 ± 6	9 ± 5
	GD100	43 ± 13	70 ± 9	16 ± 6	10 ± 5
	GD150	41 ± 11	69 ± 8	16 ± 5	12 ± 6
	PND28	54 ± 13	65 ± 7	18 ± 6	15 ± 8
3	GD70	57 ± 11*	60 ± 7	24 ± 6	5 ± 3*
	GD100	61 ± 8*	64 ± 5	23 ± 5*	7 ± 3*
	GD150	51 ± 7	63 ± 5	22 ± 7	9 ± 6*
	PND28	48 ± 20	61 ± 7	22 ± 8	15 ± 8
4	GD70	61 ± 10*	59 ± 8*	26 ± 4*	5 ± 3*
	GD100	61 ± 6*	62 ± 7*	25 ± 5*	5 ± 2*
	GD150	60 ± 9*	60 ± 9*	26 ± 7*	6 ± 3*
	PND28	70 ± 8*	60 ± 8*	31 ± 7*	5 ± 2*

[a] Mean value ± SD – for % lymphocytes plus parameters showing statistically significant changes only

* Statistically significant at $p < 0.05$, ANOVA with Dunnett's t-test

Table 7 Infant FACS Parameters

Group	Day	Parameter[a]			
		Lymphocytes (%)	$CD3^+$ (%)	$CD20^+$ (%)	$CD71^+$ (%)
1	PND28	66 ± 12	64 ± 12	28 ± 11	0.88 ± 2.18
	PND56	79 ± 9	64 ± 10	28 ± 10	0.08 ± 0.07
	PND84	77 ± 12	65 ± 9	27 ± 9	0.09 ± 0.08
2	PND28	66 ± 14	59 ± 13	33 ± 13	0.11 ± 0.12
	PND56	75 ± 17	60 ± 10	34 ± 10	0.09 ± 0.11
	PND84	78 ± 15	57 ± 10	34 ± 8	0.05 ± 0.06
3	PND28	61 ± 21	64 ± 10	26 ± 9	0.70 ± 1.73
	PND56	76 ± 12	64 ± 9	27 ± 8	0.14 ± 0.25
	PND84	77 ± 14	68 ± 6	23 ± 7	0.08 ± 0.10
4	PND28	73 ± 12	47 ± 9*	41 ± 8	4.60 ± 2.77*
	PND56	83 ± 6	50 ± 10*	41 ± 10	2.11 ± 1.86*
	PND84	81 ± 11	53 ± 12	38 ± 12	1.15 ± 1.14*

[a] Mean value ± SD – for % lymphocytes plus parameters showing statistically significant changes only

* Statistically significant at $p < 0.05$, ANOVA with Dunnett's t-test

For infants, the only significant change in lymphocyte populations was a decrease in CD3+ for group 4 at PND 28 and 56 and increase in CD71+ at these same time points. No attempt to interpret these findings is made.

F_1 behavioral evaluation: Not evaluated.

F_1 reproduction: Not evaluated

F_2 findings: Not included.

2.6.6.7 Local tolerance NA

Local tolerance was addressed in individuals toxicology studies. No specific effect of the test article was noted. This drug is administrered intravenously. Therefore, the injection sites showed some hemorrhage and other findings that were incidental to the mechanical trauma of such and injection. No other drug-specific findings were noted.

2.6.6.8 Special toxicology studies NA

2.6.6.9 Discussion and Conclusions

2.6.6.10 Tables and Figures

2.6.7 TOXICOLOGY TABULATED SUMMARY

1 Toxicology

Overview Test Article: natalizumab

Type of Study	Species and Strain	Method of Administration	Duration of Dosing	Doses (mg/kg)	GLP Compliance	Testing Facility	Study Number	Location Section
Single-Dose Toxicity								
	CD-1 mice	IV	1 day	0, 250	Yes		AL301	M4.2.3.1
	Hartley guinea pig	IC	1 day	0, 127	Yes		AL089	M4.2.3.1
Repeat-Dose Toxicity								
	CD-1 mice	IV	14 days	0, 3, 10, 30	Yes		AL105	M4.2.3.2
	Cynomolgus monkey	IV	28 days	0, 3, 10, 30	Yes		940911	M4.2.3.2
	Cynomolgus monkey	IV	28 days	0, 0.3, 3, 30	Yes		AL106	M4.2.3.2
	Cynomolgus monkey	IV	6 months	0, 3, 10, 30, 60	Yes		723-013-98	M4.2.3.2
	Cynomolgus monkey - juvenile	IV	6 months	0, 10, 30, 60	Yes		309-011-00	M4.2.3.2
	Rhesus monkey	Avonex® - IM Natalizumab - IV	28 days	Avonex® - 0, 30 µg/dose Natalizumab – 0, 30, 60	Yes		P00002-01-01	M4.2.3.2

1 Toxicology (continued)

Overview Test Article: natalizumab

Type of Study	Species and Strain	Method of Administration	Duration of Dosing	Doses (mg/kg)	GLP Compliance	Testing Facility	Study Number	Location Section
Genotoxicity								
	L5178Y TK+/- Mouse Lymphoma	In vitro	-	0, 47.8, 95.6, 191, 382, 765, 1530 µg/mL	Yes		AM002	M4.2.3.3.1
	Human Lymphocytes	In vitro	-	0, 191, 383, 765, 1530, 2040 µg/mL	Yes		AM001	M4.2.3.3.1
Carcinogenicity								
	Various cancer lines	In vitro	-	-	No		P00002-02-05	M4.2.3.4.3
	Various cancer lines	In vitro	-	-	No		P00002-03-03	M4.2.3.4.3
	SCID mouse	IP	31-52 days	5[a]	No		P00002-03-01	M4.2.3.4.3
	Nude Mouse	IP	24-45 days	5[a]	No		P00002-03-04	M4.2.3.4.3
Reproductive Toxicity								
	Hartley guinea pig	IV	28 days	0, 30	No		309-025-00	M4.2.3.5.1
	Hartley guinea pig	IV	28-75 days	0, 3, 10, 30	Yes		309-007-01	M4.2.3.5.1
	Hartley guinea pig	IV	14 days	0, 30	No		309-005-02	M4.2.3.5.1

1 **Toxicology Overview (continued)**

Overview **Test Article:** natalizumab

Type of Study	Species and Strain	Method of Administration	Duration of Dosing	Doses (mg/kg)	GLP Compliance	Testing Facility	Study Number	Location Section
	Hartley guinea pig	IV	56-109 days	0, 3, 10, 30	Yes		309-008-01	M4.2.3.5.1
	Hartley guinea pig	IV	24 days	0, 3, 10, 30	Yes		309-009-01	M4.2.3.5.2
	Hartley guinea pig	IV	48-99 days	0, 30	Yes		309-028-02	M4.2.3.5.2
	Cynomolgus monkey	IV	30 days	0.06, 0.3, 30	No		AL302	M4.2.3.5.2
	Cynomolgus monkey	IV	50 days	0, 3, 10, 30	Yes		309-012-00	M4.2.3.5.2
	Cynomolgus monkey	In vitro	-	-	No		309-028-01	M4.2.3.5.2
	Cynomolgus monkey	IV	50-164 days	0, 30	Yes		309-033-01	M4.2.3.5.3

[a] First dose 10 mg/kg, followed by 5 mg/kg for all other doses.

2 **Toxicokinetics**

Overview of Toxicokinetics Studies **Test Article:** natalizumab

Type of Study	Test System	Method of Administration	Doses (mg/kg)	GLP Compliance	Study Number	Location Section
Repeat-Dose Toxicity	CD-1 Mouse	IV	0, 3, 10, 30	Yes	AL105	M4.2.3.2
Repeat-Dose Toxicity	Cynomolgus Monkey	IV	0, 0.3, 3, 30	Yes	AL106	M4.2.3.2
Repeat-Dose Toxicity	Cynomolgus Monkey	IV	0, 3, 10, 30, 60	Yes	723-013-98	M4.2.3.2
Repeat-Dose Toxicity	Cynomolgus Monkey	IV	0, 10, 30, 60	Yes	309-011-00	M4.2.3.2
Repeat-Dose Toxicity	Rhesus Monkey	IV	0, 30, 60	Yes	P00002-01-01	M4.2.3.2
Carcinogenicity	SCID Mouse	IP	0, 5	No	P00002-03-01	M4.2.3.4.3
Carcinogenicity	Nude Mouse	IP	0, 5	No	P00002-03-04	M4.2.3.4.3
Reproductive Toxicity	Hartley Guinea Pig	IV	0, 3,10, 30	Yes	309-007-01	M4.2.3.5.1
Reproductive Toxicity	Hartley Guinea Pig	IV	0, 3,10, 30	Yes	309-008-01	M4.2.3.5.1
Reproductive Toxicity	Hartley Guinea Pig	IV	0, 3,10, 30	Yes	309-009-01	M4.2.3.5.2
Reproductive Toxicity	Hartley Guinea Pig	IV	0, 30	Yes	309-028-02	M4.2.3.5.2
Reproductive Toxicity	Cynomolgus Monkey	IV	0.006, 0.3, 30	Yes	AL302	M4.2.3.5.2
Reproductive Toxicity	Cynomolgus Monkey	IV	0, 3, 10, 30	Yes	309-012-00	M4.2.3.5.2
Reproductive Toxicity	Cynomolgus Monkey	IV	0, 30	Yes	309-033-01	M4.2.3.5.3

3 Toxicokinetic Profiles

Test Article: natalizumab

Study Number	Type of Study	Test System	Doses (mg/kg)	Route/Dosing Frequency	Dosing Duration (Days)	Location of TK Profile Section 10 of Report 2.6.6, Toxicology Written Summary
AL105 M4.2.3.2	Repeat-Dose Toxicity	CD-1 Mouse	0, 3, 10, 30	IV/Daily	14	Table 25
AL106 M4.2.3.2	Repeat-Dose Toxicity	Cynomolgus Monkey	0, 0.3, 3, 30	IV/Alternate days	28	Table 26
723-013-98 M4.2.3.2	Repeat-Dose Toxicity	Cynomolgus Monkey	0, 3, 10, 30, 60	IV/Weekly	176	Table 27
309-011-00 M4.2.3.2	Repeat-Dose Toxicity	Cynomolgus Monkey	0, 10, 30, 60	IV/Weekly	176	Table 28
P00002-01-01 M4.2.3.2	Repeat-Dose Toxicity	Rhesus Monkey	0, 30, 60	IV/Weekly	28	Table 29
P00002-03-01 M4.2.3.4.3	Carcinogenicity	SCID Mouse	0, 5	IP/Twice a Week	32, 53	Table 30
P00002-03-04 M4.2.3.4.3	Carcinogenicity	Nude Mouse	0, 5	IP/Twice a Week	25, 46	Table 31
309-007-01 M4.2.3.5.1	Reproductive Toxicity	Hartley Guinea Pig	0, 3,10, 30	IV/Alternate days	28-75	Table 32
309-008-01 M4.2.3.5.1	Reproductive Toxicity	Hartley Guinea Pig	0, 3,10, 30	IV/Alternate days	56-109	Table 33
309-009-01 M4.2.3.5.2	Reproductive Toxicity	Hartley Guinea Pig	0, 3,10, 30	IV/Alternate days	24	Table 34
309-028-02 M.4.2.3.5.2	Reproductive Toxicity	Hartley Guinea Pig	0, 30	IV/Alternate days	48-99	Table 35

3 Toxicokinetic Profiles (continued)

Test Article: natalizumab

Study Number	Type of Study	Test System	Doses (mg/kg)	Route/Dosing Frequency	Dosing Duration (Days)	Location of TK Profile Section 10 of Report 2.6.6, Toxicology Written Summary
AL302 M4.2.3.5.2	Reproductive Toxicity	Cynomolgus Monkey	0.006, 0.3, 30	IV/Alternate days	30	Table 36
309-012-00 M4.2.3.5.2	Reproductive Toxicity	Cynomolgus Monkey	0, 3, 10, 30	IV/Alternate days	50	Table 37
309-033-01 M.4.2.3.5.3	Reproductive Toxicity	Cynomolgus Monkey	0, 30	IV/Alternate days	50-164	Table 38

OVERALL CONCLUSIONS AND RECOMMENDATIONS

Conclusions:
Natalizumab was generally well tolerated under the conditions of the toxicology studies included in this BLA. The major toxicities are results of the known pharmacological action of the molecule. During exposure to natalizumab during pregnancy, transplacental transfer may occur resulting in fetal exposure of approximately 30% of maternal levels. This exposure does cause effects in the fetuses including hematologic findings that are attributable to the pharmacological action of the drug. Under the conditions in the reproductive toxicology studies included in this BLA, the fetal hematological effects appear to be reversible.

Unresolved toxicology issues (if any):

The final study report for study number 309-033-01 remains outstanding. This review is based on preliminary/interim data available at the time of the BLA submission. A pot-marketing commitment has been established for submission of the final report for study 309-033-01.

An audit of the reproductive toxicology studies (specifically study #309-008-01) to determine if dosing of the female guinea pigs in that study was performed as specified in the protocol. This recommendation is due to the finding at initiation of study number 309-007-01 (male guinea pig fertility study) that several male guinea pigs died suddenly after the initial dosing. After investigations into the cause of death, it was determined that the male guinea pigs had died of anaphylaxis due to drug hypersensitivity. (See study number 309-005-01). The male guinea pigs that died, were supposedly naïve, but had been used in study 309-008-01 to cohabit with the female guinea pigs that were being dosed during implantation and gestation. There is no explanation given how those male guinea pigs were exposed to study drug. Nor was any explanation/clarification given of potential impact of accidental/erroneous dosing of the males in study 309-008-01 may have had on dosing of the females in that study. It is clear from the positive findings in study 309-008-01 as well as the other reproductive toxicology studies included in the BLA, that exposure to the study drug during pregnancy can have a negative effect on fertility and fetal clinical condition. Therefore, the potential problem with performance of study 309-008-01 should have no effect approvability of the drug. A pregnancy category C rating has been assigned and a warning regarding potential ill effects of natalizumab exposure during pregnancy will stand. The audit of the non-clinical studies in question will be carried out independent of the approval process.

Recommendations:

Non-clinical toxicology studies indicated that natalizumab is generally well tolerated. The toxicities observed were primarily extensions of the known pharmacologic activity of the drug, appear to be reversible and can be monitored in patients. The indication for this biological therapeutic agent is for treatment of relapsing multiple Sclerosis, a serious degenerative disease. There are no non-clinical findings that should deter approval.

Recommendation for nonclinical studies:

No further non-clinical studies are required at this time for the indication, route of administration and dosing regimen currently under consideration. The complete study report pre-and postnatal developmental toxicology (Study # 309-033-01) is not complete. At the time of BLA filing, only data from the in-life portions of the study were available. The gross necropsy data, organ weights, immunohistochemistry and histology data are yet to be submitted.

D. Recommendations on labeling

Non-clinical reproductive toxicology studies demonstrate that treatment of natalizumab has potential for reducing fertility through impairment of embryonic implantation. In monkeys and guinea pigs a small tendency toward postimplantation loss and decreased fetal survival was noted. In monkeys and guinea pigs, natalizumab was found to undergo transport across the placenta and fetal drug levels were roughly 30% of maternal levels. Infants exposed to natalizumab before birth were born with hematologic characteristic of natalizumab exposure (increased WBC, nRBC, increased circulating lymphocytes). Therefore, Pregnancy category C is recommended for this drug.

Signatures (optional):

Reviewer Signature _____

Supervisor Signature_____ Concurrence Yes ___ No ___

APPENDIX/ATTACHMENTS

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH

PHARMACOLOGY/TOXICOLOGY REVIEW AND EVALUATION

STN BLA NUMBER:	**125104**
SERIAL NUMBER:	**000**
DATE RECEIVED BY CENTER:	**5/23/04**
DRUG NAME:	**natalizumab (TYSABRI™)**
INDICATION:	**treatment of relapsing-remitting multiple sclerosis**
SPONSOR:	**Biogen/IDEC** **14 Cambridge Center** **Cambridge, MA 02142**
DOCUMENTS REVIEWED:	**E-CTD BLA Submission; Modules 2 and 4**
REVIEW DIVISION:	**Division of Therapeutic Biological Internal Medicine Products (HFD-108)**
PHARM/TOX REVIEWER:	**Anne M. Pilaro, Ph.D.**
PHARM/TOX SUPERVISOR:	**M. David Green, Ph.D.**
DIVISION DIRECTOR:	**Marc K. Walton, M.D., Ph.D.**
PROJECT MANAGER:	**Beverly Conner, Pharm.D.** **Cathleen Michaeloski, B.S.N., M.P.H.**

Date of review submission to Division File System (DFS):

TABLE OF CONTENTS

EXECUTIVE SUMMARY

I. Recommendations

A. Recommendation on approvability

The Biologics Licensing Application STN BLA #125104/0 is approvable based on the data contained in the preclinical pharmacology and pharmacokinetic sections of the original submission. Toxicities of natalizumab in the reviewed pharmacology studies were limited to increases in circulating total leukocytes and differential lymphocyte, monocytes, eosinophils, and basophil counts. These effects are extensions of the pharmacologic activity of the product, were reflected in the clinical studies, and may be monitored appropriately in the clinical setting.

B. Recommendation for nonclinical studies

There are no recommendations at the present time for the sponsor to conduct and submit for FDA review any additional nonclinical, pharmacology studies with natalizumab in support of either its safety or efficacy.

C. Recommendations on labeling

Modifications to the CLINICAL PHARMACOLOGY section of the label, including revision of the sponsor's language regarding the specificity of natalizumab for the α4 chain of the α-integrin family, the role of α4 integrin in binding to various extracellular matrix proteins, and the potential mechanism of action of natalizumab in multiple sclerosis have been communicated to and accepted by the sponsor. Copies of the final, revised language for these sections of the labeling are included as Appendix 1 to this review.

II. Summary of nonclinical findings

A. Brief overview of nonclinical findings

Natalizumab (AN100226, AN100226m) was evaluated for pharmacologic activity both *in vitro* and *in vivo* using α4 integrin-expressing human cell lines, peripheral blood mononuclear cells from various species, and in an experimental allergic encephalomyelitis model of multiple sclerosis in guinea pigs. Tissue binding studies demonstrated that AN100226 or AN100226m cross-reactivity was limited to lymphoid tissues, including lymph node, spleen, thymus, tonsil, and gut-associated lymphoid tissue in both adult and fetal human and cynomolgus monkey samples. Binding of natalizumab to uterine basal endometrial cells was observed in samples from human donors, and low levels of cross-reactivity in prostate from cynomolgus monkeys, but not human donors were also observed. There was no cross-reactivity of natalizumab with brain tissue from either adult or fetal human or cynomolgus monkey samples. Flow cytometric evaluation of natalizumab binding to peripheral blood mononuclear cells showed cross-reactivity with cells from cynomolgus or Rhesus macaque, dog, swine, ferret, and guinea pig at levels that were comparable to binding with human cells, but no detectable binding to peripheral blood leukocytes from mouse, rat, marmoset, rabbit, or gerbil. *In vitro* pharmacology studies showed that binding of human cell lines expressing α4 integrin to activated endothelial cells, or to cell lines transfected with the human gene for the vascular cell adhesion molecule-1 (VCAM-1) counter-ligand for α4 integrin could be inhibited by incubation with AN100226m or AN100226. Treatment of guinea pigs with natalizumab in an established, experimental allergic

encephalomyelitis (EAE) model was associated with marked decreases in leukocyte infiltration into brain tissue. Reduction of cerebral edema and plaque formation as detected by magnetic resonance imaging and reversal of the clinical signs of illness were also observed in these studies, and these effects were diminished following discontinuation of natalizumab treatment. Pharmacokinetic profiles of AN100226 and AN100226m following single, or repeat intravenous administrations in cynomolgus monkeys, or male and pregnant female guinea pigs demonstrated dose-related, although non-linear increases in C_{max} and $AUC_{0\text{-last}}$, elimination half-life and mean residence times, and decreases in systemic clearance. The volume of distribution of natalizumab at steady state was approximately equal to or slightly greater than the plasma space, suggesting limited distribution of the antibody to the extracellular fluid. Treatment of Rhesus monkeys with four weekly administrations of natalizumab, either alone or in combination with the recombinant human interferon-beta 1a (IFN-β) AVONEX® did not result in any alteration of the pharmacodynamic, pharmacokinetic, or immunogenicity profiles of either AN100226 or AVONEX®, as compared to values obtained for monkeys treated with either product alone. Anti-natalizumab antibody development was observed in both the cynomolgus monkey and guinea pig studies, with both incidence and titers that were inversely related to the dose of AN100226 or AN100226m administered.

B. Pharmacologic activity

Integrins are a family of heterodimeric, transmembrane receptor proteins that are involved in a variety of physiological processes, including inflammation. To date, over 15 different α- and 8 different β-subunits have been identified. Each specific integrin receptor is formed from one α- and one β-subunit, of which there are at least 20 different heterodimeric combinations published to date. Natalizumab (AN100226, AN100226m) binds to the α4 subunit of the α4β1 and α4β7 integrins constitutively expressed on the surface of all leukocytes, except neutrophils. In multiple sclerosis (MS), lesions in the white matter of the brain are believed to occur when activated inflammatory leukocytes, including T lymphocytes and monocytes, cross the endothelial cells of the blood-brain barrier, and migrate into the parenchyma. Although the exact mechanism(s) by which natalizumab exerts its effects in MS have not been identified, interference of binding of α4 integrins to the counter-ligand VCAM-1 may prevent the recruitment and activation of inflammatory lymphocytes and monocytes across the blood-brain barrier. Preclinical pharmacology studies conducted *in vitro* using the human T cell lines Jurkat and Ramos, which both express the α4β1 integrin complex, and mouse L-cells that were transfected with the human gene for α4 integrin demonstrated that either natalizumab or the parental murine monoclonal antibody AN100226m could bind to the cell surface with high affinity. Both AN100226m and natalizumab could bind to peripheral blood mononuclear cells from human donors, cynomolgus and Rhesus macaques, and guinea pigs with approximately equal affinity, as determined by flow cytometry. The K_d for natalizumab binding to human, Rhesus, and cynomolgus monkey lymphocytes was 0.07 μg/ml, and a K_d of 0.04 μg/ml was observed for AN100226 binding to guinea pig lymphocytes. No binding of either natalizumab or AN100226m to lymphocytes from mouse, rat, gerbil, hamster, rabbit, ferret, or marmoset was detected. *In vitro* binding of human Jurkat or Ramos T cells to TNF-α activated, cultured rat brain or human umbilical vein endothelial cells, or to cell lines transfected with the human gene for VCAM-1 could be inhibited in a dose-related fashion by AN100226m or AN100226. Tissue binding studies to a panel of frozen sections from adult and fetal human and cynomolgus monkey tissues, and to fetal Rhesus monkey tissue sections revealed that natalizumab staining was localized to tissues of lymphoid origin, with the most intense staining present (2^+-3^+) in the lymph nodes, spleen, thymus, and germinal centers of tonsillar tissue, in the gut-associated lymphoid tissue present in the small and large intestines, and in occasional interstitial lymphocytes present in mammary, lung, parathyroid,

and stomach samples. The pattern of tissue binding of AN100226 or AN100226m in the cynomolgus monkey was similar to that observed in the human tissues, with two exceptions. One of two samples of human uterus showed strong (3^+) staining localized to the basilar endometrial cell layer, which was not observed in any of the cynomolgus or Rhesus monkey samples. In cynomolgus monkeys, weak but detectable natalizumab binding ($\pm$ to 1^+) was observed in samples of prostate tissue, with no comparative activity noted in the human samples. *In vivo* studies of AN100226 in an experimental guinea pig EAE model of MS showed that treatment with 3 mg natalizumab (approximately 12 mg/kg) s/c every other day for 2 or 3 injections after clinical signs of illness appeared could markedly reduce, or reverse the clinical signs of disease. Histopathological evaluation of brain sections from AN100226-treated, EAE guinea pigs showed marked reductions in inflammatory leukocyte infiltrations, edema, and tissue damage when compared to control animals injected with vehicle alone. Magnetic resonance imaging of these animals showed treatment-related decreases in cerebral edema and plaque formation as compared to the control group, which was reversible within 72 hours following discontinuation of treatment. Taken together, these data suggest that the guinea pig and the cynomolgus monkey are species that are pharmacologically responsive to AN100226, and supports their use for additional pharmacokinetic and toxicology testing. Single-dose pharmacokinetic studies of AN100226m, AN100226, or natalizumab manufactured at different stages of product development showed dose-related, although proportionally non-linear increases in C_{max}, $AUC_{0\text{-last}}$, mean residence time, and elimination half-lives, and decreased clearance values that were suggestive of a saturable clearance mechanism(s) in both cynomolgus macaques and male and pregnant and non-pregnant female guinea pigs. Mean elimination half-life values for natalizumab ($\pm$ S.D.) following a single, intravenous dose of 0.3, 3.0, or 30 mg/kg in cynomolgus monkeys were 8.2 ± 3.8, 58.0 ± 24.2, and 73.8 ± 23.4 hours, respectively. In the guinea pig, the mean elimination half-lives were 18.8 ± 5.3, 36.9 ± 7.9, and 73.0 ± 24.1 hours following a single, intracardiac injection of 1, 3, or 8 mg/kg respectively, of AN100226. Both species showed comparable elimination half-life values after a dose of 3.0 mg/kg natalizumab, with a range of 58.0 to 80.1 hours in the monkey, and a mean value of 36.9 ± 7.9 hours in the guinea pig after intra-cardiac injection. Combination treatment of Rhesus macaques with weekly doses of 30 or 60 mg/kg AN100226 and 30 µg/animal AVONEX® had no remarkable effects on the pharmacodynamic, pharmacokinetic, or immunogenic profiles of either product, as compared to animals receiving either agent alone. Antibodies to natalizumab were detected in all test species in both single and repeat-dose studies, in an inverse relationship for both incidence and titer to the dose of AN100226 or AN100226m administered. Anti-natalizumab antibody levels in serum tended to decrease with longer duration of treatment-free recovery periods. Taken together, these data demonstrate that natalizumab is pharmacologically active in both the cynomolgus monkey and in an experimental guinea pig EAE model of MS, demonstrates pharmacokinetic profiles in both species that are dose-related although non-linear, and is capable of inducing an antibody response in the host directed at natalizumab even following a single administration, which may relate to its long elimination half-life and subsequent duration of exposure following intravenous injection.

C. Nonclinical safety issues relevant to clinical use

Increases in total peripheral blood leukocyte counts, and in differential lymphocyte, monocytes, eosinophils, and basophil counts were the only toxicities noted with AN100226m or AN100226 treatment in the studies included in this review. These findings are not unexpected given the proposed mechanism of natalizumab action, which is inhibition of leukocyte migration out of the vascular compartment into tissue sites, and are considered an exaggeration of the pharmacologic activity of the product rather than a true toxicity. Similar increases in peripheral blood mononuclear cells, but not neutrophils were observed in the clinical studies of natalizumab in multiple sclerosis, and were reversible upon discontinuation of treatment.

2.6 PHARMACOLOGY/TOXICOLOGY REVIEW

2.6.1 INTRODUCTION AND DRUG HISTORY

STN BLA number: 125014/0
Review number: 002
Sequence number/date/type of submission: 000/May 23, 2004/original licensing application
Information to sponsor: Yes ( ) No (**X**)
Sponsor and/or agent: Biogen/IDEC Corporation
Manufacturer for drug substance: Biogen/IDEC Corporation
5000 Davis Drive
Research Triangle Park, NC 27709

Reviewer name: Anne M. Pilaro, Ph.D.
Division name: Division of Therapeutic Biological Internal Medicine Products
HFD #: 108
Review completion date: November 17, 2004

Drug:

Trade name: TYSABRI™
Generic name: natalizumab
Code names: AN100226 (humanized version), AN100226m (initial murine version), BG00002, Antegren (proposed trade name rejected by DMETS)
Chemical name: not available
CAS registry number: not available
Molecular formula/molecular weight: molecular formula not available; MW 149 kD
Structure: Natalizumab is a monoclonal antibody of the IgG4κ subclass, and is

PH-3

1 page(s) have been removed because it contains trade secret and/or confidential information that is not disclosable

Relevant INDs/NDAs/DMFs:
BB IND #6895 (sponsored by Elan Pharmaceuticals, for treatment of multiple sclerosis); BB IND

Drug class: monoclonal antibody; immunomodulatory

Indication: Natalizumab is indicated for treatment of patients with relapsing forms of multiple sclerosis, to reduce the frequency of clinical exacerbations.

Clinical formulation: Natalizumab is formulated for clinical use at a concentration of 20 mg of antibody/ml, in buffer containing sodium chloride, sodium phosphate, monobasic, monohydrate, dibasic sodium phosphate, heptahydrate, polysorbate 80, and sterile Water for Injection, USP, at a pH of 6.1.

Route of administration: intravenous infusion

Disclaimer: Tabular and graphical information are constructed by the reviewer unless cited otherwise.

Studies reviewed within this submission:
Pharmacology Studies:
Primary pharmacodynamics

1. Affinity of Antegren for α4 integrin expressed by circulating lymphocytes from human, guinea pig, cynomolgus, and Rhesus monkeys. Study #309-001-03.
2. Specificity of AN100266m for α4 integrin. Study #309-002-04.
3. Binding of Antegren (natalizumab) to mouse lymphocytes. Study #309-025-03.
4. Guinea pig EAE reversal study – Comparison of Antegren and GG5/3. Measurement of EAE reversal (body weight and clinical score) and histological analysis of infiltrating cells. Study #310-1-A.
5. Efficacy of AN100226 in guinea pig experimental allergic encephalomyelitis after subcutaneous administration. Study #AL078 (laboratory report #94124).
6. A monoclonal antibody to α4 integrin suppresses and reverses active experimental allergic encephalomyelitis. Kent S.J. *et al.*, *J. Neuroimmunol.*, **58**:1-10 (1995).
7. A monoclonal antibody to α4-integrin reverses the MR-detectable signs of active experimental allergic encephalomyelitis in the guinea pig. Kent, S.J. *et al.*, *J. MRI*, 5:535-540.
8. Characterization of AN100226 and AN100226m as inhibitory antibodies against human alpha-4 integrin. Study #PC032.
9. Expression of α4 integrin on lymphocytes isolated from healthy volunteers and from patients with multiple sclerosis. Study #PC100.

Secondary pharmacodynamics

1. *In vitro* evaluation of the immunomodulatory potential of anti-α4 integrin antibody. Study #PC028.

Safety Pharmacology Studies:

1. A cardiovascular profile study following a single intravenous infusion of AN100226 in the conscious beagle dog. Study #AL107.

Pharmacokinetic Studies:

1. A single-dose pharmacokinetic intravenous infusion study of Antegren in cynomolgus monkeys – comparison of [redacted] Biogen processes. Study #309-003-01.
2. Pharmacokinetic study of Antegren (AN100226, natalizumab) in guinea pigs. Study #309-010-01.
3. A single-dose pharmacokinetics study with intravenous infusion of Antegren in female cynomolgus monkeys – comparison of [redacted] processes. Study #723-004-98.
4. A single-dose pharmacokinetic study with intravenous infusion of Antegren™ in female cynomolgus monkeys. Study #723-012-98.
5. Pharmacokinetics of AN100226 in male and female guinea pigs after intracardiac administration. Study #AL077.
6. Pharmacokinetics of AN100226 in the cynomolgus monkey. Study #AL109.
7. A single dose pharmacokinetic study with intravenous infusion of AN100226 in female cynomolgus monkeys. Study #AL-300 ([redacted] Study #960711).
8. BG00002: A single-dose intravenous pharmacokinetic study of natalizumab, [redacted] commercial process material versus natalizumab Biogen resupply material in female cynomolgus monkeys. [redacted] Study #1348-87 (Sponsor Study #P00002-02-01).
9. Pharmacokinetic report. Study #PD03-09 (Biogen studies #P00002-02-02, #P00002-02-03, #P00002-02-04, and #P00002-03-02).

Pharmacokinetic Drug Interaction Studies:

1. Avonex®/Antegren: A four-week combination study of Antegren administered intravenously and Avonex® administered intramuscularly in the Rhesus monkey, followed by an eight-week recovery. Study #P00002-01-01.

Tissue Distribution Studies:

1. Cross reactivity of mouse monoclonal antibody 21-6 (AN100,226M) with human tissues. Study #IM050.
2. Cross-reactivity of humanized monoclonal antibody AN100226 with human tissues. Study #PC002 [redacted] Study #IM106).
3. Cross reactivity of humanized monoclonal antibody AN100226 with cynomolgus monkey tissues and guinea pig heart. Study #PC001 ([redacted] Study #IM179).
4. Cross-reactivity of Antegren™ (natalizumab) with fetal and perinatal tissues and fetal Rhesus and cynomolgus monkey tissues. Study #IM342.

Studies not reviewed within this submission: No additional pharmacology studies have been identified as submitted to the IND that are not included in the BLA submission. There are a number of articles from the open literature included in the references to the pharmacology section of the BLA submission, that document the effects of natalizumab and other anti-integrin antibody preparations on leukocyte adhesion, cytokine response and effector cell function. These articles were not included as part of the BLA review, unless specifically cited in the primary

pharmacodynamics module of the [redacted] submission. A list of these references is provided as Appendix 2 of this review.

The toxicology and toxicokinetic data for natalizumab are reviewed separately by Barbara J. Wilcox, Ph.D., in the Division of Biological Internal Medicine Products, ODE-VI, CDER.

2.6.2 PHARMACOLOGY

2.6.2.1 Brief summary

Integrins are a family of heterodimeric, transmembrane receptor proteins that are involved in a variety of physiological processes, including inflammation. To date, over 15 different α- and 8 different β-subunits have been identified. Each specific integrin receptor is formed from one α- and one β-subunit, of which there are at least 20 different heterodimeric combinations published to date. Natalizumab (AN100226, AN100226m) binds to the α4 subunit of the α4β1 and α4β7 integrins constitutively expressed on the surface of all leukocytes, except neutrophils.

In multiple sclerosis (MS), lesions in the white matter of the brain are believed to occur when activated inflammatory leukocytes, including T lymphocytes and monocytes, cross the endothelial cells of the blood-brain barrier, and migrate into the parenchyma. Although the exact mechanism(s) by which natalizumab exerts its effects in MS have not been identified, interference of binding of α4 integrins to the counter-ligand VCAM-1 may prevent the recruitment and activation of inflammatory lymphocytes and monocytes across the blood-brain barrier. Preclinical pharmacology studies conducted *in vitro* using the human T cell lines Jurkat and Ramos, which both express the α4β1 integrin complex, and mouse L-cells that were transfected with the human gene for α4 integrin demonstrated that either natalizumab or the parental murine monoclonal antibody AN100226m could bind to the cell surface with high affinity. Both AN100226m and natalizumab could bind to peripheral blood mononuclear cells from human donors, cynomolgus and Rhesus macaques, and guinea pigs with approximately equal affinity, as determined by flow cytometry. The K_d for natalizumab binding to human, Rhesus, and cynomolgus monkey lymphocytes was 0.07 μg/ml, and a K_d of 0.04 μg/ml was observed for AN100226 binding to guinea pig lymphocytes. No binding of either natalizumab or AN100226m to lymphocytes from mouse, rat, gerbil, hamster, rabbit, ferret, or marmoset was detected. *In vitro* binding of human Jurkat or Ramos T cells to TNF-α activated, cultured rat brain or human umbilical vein endothelial cells, or to cell lines transfected with the human gene for VCAM-1 could be inhibited in a dose-related fashion by AN100226m or AN100226.

In vivo studies of AN100226 in an experimental guinea pig EAE model of MS showed that treatment with 3 mg/kg natalizumab s/c every other day for 2 to 5 injections after clinical signs of illness appeared could markedly reduce, or reverse the clinical signs of disease. Histopathological evaluation of brain sections from AN100226-treated, EAE guinea pigs showed marked reductions in inflammatory leukocyte infiltrations, edema, and tissue damage when compared to control animals injected with vehicle alone. Magnetic resonance imaging of these animals showed treatment-related decreases in cerebral edema and plaque formation as compared to the control group, which was reversible within one week following discontinuation of treatment. Taken together, these data suggest that the guinea pig and the cynomolgus monkey are species that are pharmacologically responsive to AN100226, and supports their use for additional pharmacokinetic and toxicology testing.

2.6.2.2 Primary pharmacodynamics

Mechanism of action: Natalizumab binds to the α4β1 integrin expressed on the surface of all leukocytes except neutrophils, and inhibits the α4-integrin mediated adhesion of leukocytes to their counter-receptors. The receptors for the α4 family of integrins include VCAM-1, which is expressed on activated vascular endothelium, and the mucosal addressin cell adhesion molecule-1 (MadCAM-1) present on mucosal endothelial cells. Disruption of these molecular interactions prevents attachment and migration of leukocytes across the endothelium into inflamed parenchymal tissue. *In vitro*, anti-α4 integrin antibodies that recognize the same epitope on VCAM-1 as natalizumab also block α4-mediated cell binding to ligands such as osteopontin and an alternatively spliced domain of fibronectin, connecting segment-1 (CS-1). *In vivo*, natalizumab may further act to inhibit the interaction of α4-expressing leukocytes with their ligand(s) in the extracellular matrix and on parenchymal cells, thereby inhibiting further recruitment and inflammatory activity of activated immune cells.

Drug activity related to proposed indication: The specific mechanism(s) by which natalizumab exerts its effects in MS have not been fully defined. In MS, lesions are believed to occur when activated inflammatory cells, including T-lymphocytes, cross the blood-brain barrier. Leukocyte migration across the blood-brain barrier involves interaction between adhesion molecules on inflammatory cells, and their counter-receptors present on endothelial cells of the vessel wall. The clinical effect of natalizumab in multiple sclerosis may be secondary to blockade of the molecular interaction of α4β1-integrin expressed by inflammatory cells with VCAM-1 on vascular endothelial cells, and with CS-1 and/or osteopontin expressed by parenchymal cells in the brain. Preclinical data from an EAE model of MS in guinea pigs have demonstrated significant reduction of leukocyte migration into brain parenchyma, and reduction of plaque formation detected by magnetic resonance imaging (MRI) following repeated administration of natalizumab.

The following studies were included in the BLA submission as support for the primary pharmacodynamic activity of natalizumab, both *in vitro* and *in vivo*.

Study title: Affinity of Antegren for α4 integrin expressed by circulating lymphocytes from human, guinea pigs, cynomolgus and Rhesus monkeys

Key findings: Natalizumab (Antegren, AN100226) binds to human, guinea pig, and Rhesus and cynomolgus monkey lymphocytes with approximately equal affinity, supporting the utility of these models for additional pharmacologic, toxicological, and pharmacokinetic testing.

Study #: 309-001-003

Volume # and page #: EDR file: STN BLA 125104\000\module4\primarypharm\309-001-003.pdf

Conducting laboratory and location: Elan Pharmaceuticals, 800 Gateway Boulevard, South San Francisco, CA 94080

Date of study initiation: not specified (final report dated January 9, 2003)

GLP compliance: no

QAU statement: yes ( ) no (X)

Drug, lot #, and % purity: AN100226 (natalizumab), 20 mg/ml stock; lot #E23001; % purity not specified in final study report

Methods: Peripheral blood samples (0.1 ml each) obtained from three individual cynomolgus or Rhesus macaques, guinea pigs, and healthy female human volunteer subjects

Results: The humanized version of natalizumab, AN100226 bound to peripheral blood lymphocytes from human, monkey, and guinea pigs with approximately equal affinity. However, cynomolgus monkey samples tended to show higher levels of natalizumab binding as compared to the other test animal species, at concentrations of 0.11 µg/ml and above. The results from this experiment are shown in Figure 2 below, which was provided by the sponsor in the final study report.

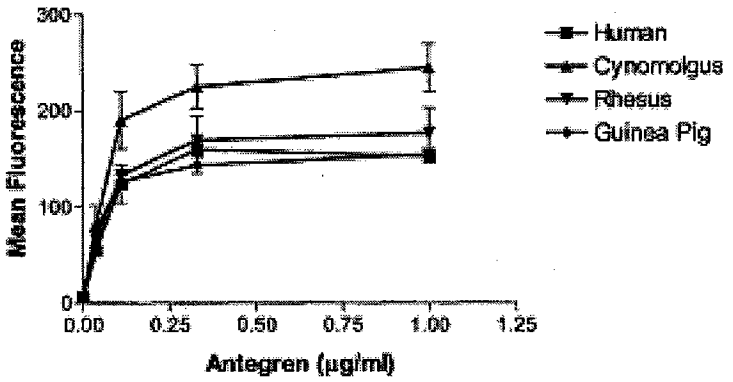


Figure 2. Antegren binding to peripheral blood lymphocytes from different species (data represent the mean ± S.D. for three individual samples).

Dissociation constants (K_d values) were calculated from the concentration/response curve using the GraphPad PRISM software package, version 3.02. The K_d for Antegren binding to human leukocytes was approximately 0.07 µg/ml, which was similar to the value of 0.04 – 0.05 µg/ml obtained previously in patients with multiple sclerosis (please see review of Study #PC001, below). Similar values were obtained for the test animal species, with both cynomolgus and Rhesus monkeys having a calculated K_d of 0.07 µg/ml, and a K_d of 0.04 µg/ml observed for the guinea pig.

Study conclusion: Natalizumab binds to peripheral blood lymphocytes from human, guinea pigs, Rhesus, and cynomolgus macaques, as determined by flow cytometry. Although the K_d values were similar between human lymphocytes and all test animal species, binding of AN100226 in cynomolgus monkeys was increased over that observed in the other species. Taken together, these data demonstrate that natalizumab binds to lymphocytes from guinea pig and cynomolgus monkey with approximately equal affinity as to human cells, and supports the use of these test animal species for further evaluation of the safety of the product.

Study title: Specificity of AN100266m for α4 integrin.

Key findings: The parental murine monoclonal antibody AN100226m bound only to human T lymphoid cells expressing the α4β1 integrin, and not to human cells expressing the α5, α9, or β5 integrins.

Study #: 309-002-04

Volume # and page #: EDR file: STN BLA 125104\000\module4\primarypharm\309-002-04.pdf

Conducting laboratory and location: Elan Pharmaceuticals, 800 Gateway Boulevard, South San Francisco, CA 94080

Date of study initiation: not specified (final report dated March 25, 2004)

GLP compliance: no

QAU statement: yes ( ) no (X)

Drug, lot #, and % purity: AN100226m, concentration and formulation, percent purity not specified in the final report

Methods: The specificity of the murine monoclonal antibody AN100226m (Antegren) for α4 integrins was demonstrated by flow cytometry, using human cell lines expressing α4

Study title: Binding of Antegren (natalizumab) to mouse lymphocytes.

Key findings: Antegren (natalizumab) did not bind to lymphocytes isolated from mouse blood, but did bind to guinea pig peripheral blood lymphocytes, as determined by flow cytometry.

Study #: 309-025-03

Volume # and page #: EDR file: STN BLA 125104\000\module4\primarypharm\309-025-03.pdf

Conducting laboratory and location: Elan Pharmaceuticals, 800 Gateway Boulevard, South San Francisco, CA 94080

Date of study initiation: not specified (final report dated October 28, 2003)

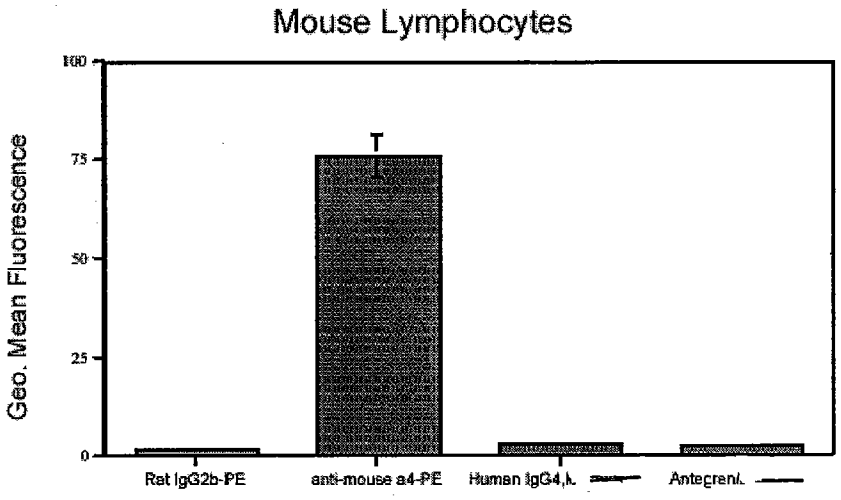
GLP compliance: no

QAU statement: yes ( ) no (X)

Drug, lot #, and % purity: AN100226 (natalizumab), lot #E23001; % purity not specified in final study report

Methods: Peripheral blood was obtained by cardiac puncture from 3 female Swiss-Webster mice and 3 female guinea pigs. The humanized form of natalizumab, AN100226 (lot #E23001) was used as the primary antibody for detection of α4 integrin expression, and an irrelevant, human IgG4κ was used as an isotype-matched antibody control.

Results: Natalizumab did not bind to mouse peripheral blood lymphocytes, while binding to lymphocytes from guinea pigs was observed as previously described. Figure 4, below was provided by the sponsor in the final study report. Mean fluorescent intensity (MFI) for AN100226 binding to guinea pig lymphocytes was 172.0 ± 9.1, as compared to 2.9 ± 0.1 for natalizumab binding to mouse lymphocytes. Mouse α4 integrin was detectable using the rat anti-mouse antibody R1-2, with a MFI of 76.1 ± 5.4, while both the PE-conjugated human IgG4κ and the rat IgG2b control antibodies bound with MFI of < 4.



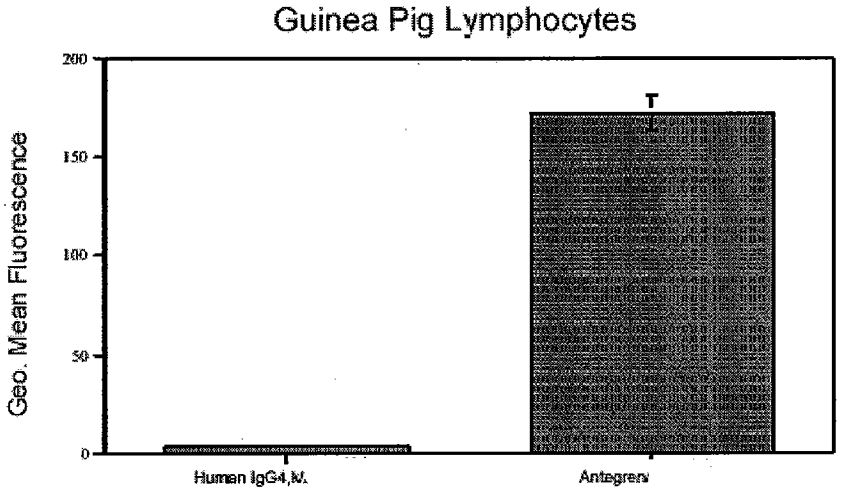


Figure 4. Natalizumab (Antegren, AN100226) binding to lymphocytes from mouse and guinea pig.

Study conclusion: Natalizumab did not bind α4 integrin expressed on murine lymphocytes. As previously demonstrated, there was a high degree of AN100226 binding detected in peripheral blood lymphocytes isolated from female guinea pigs. Therefore, natalizumab does not cross-react with murine α4 integrin, and the mouse is not a pharmacologically relevant species.

Study title: Guinea pig EAE reversal study – Comparison of Antegren and GG5/3. Measurement of EAE reversal (body weight and clinical score) and histological analysis of infiltrating cells.

Key findings: Treatment of guinea pigs by s/c injection with 3 mg/kg natalizumab (AN100226) produced during process scale-up to was able to protect guinea pigs from clinical signs of illness, and prevent infiltration of brain and spinal cord by inflammatory leukocytes in an EAE experimental model of MS.

Study #: 310-1-A

Volume # and page #: EDR file: STN BLA 125104\000\module4\primarypharm\310-1-a.pdf
Conducting laboratory and location:

Date of study initiation: not specified (final report dated October 26, 1998)
GLP compliance: no
QAU statement: yes ( ) no (X)
Drug, lot #, and % purity: AN100226 (humanized version; natalizumab), lot #C0237, 5.4 mg/ml; purity IgG as heavy and light chains, as detected by SDS-PAGE gel electrophoresis under reducing and non-reducing conditions; monomeric IgG as detected by HPLC (certificate of analysis was included as an appendix to the final study report)

Methods: The ability of natalizumab produced during process scale-up to ameliorate the clinical signs and histopathology findings in a guinea pig model of experimental allergic encephalitis (EAE) was compared to that of the anti-α4 integrin monoclonal antibody, GG5/3. Forty-five female Hartley strain guinea pigs were immunized with homogenized brain tissue from isogenic guinea pigs emulsified with Freund's complete adjuvant on Study Day 0. Clinical scores and body weights were measured on Study Days 0, 3, 5, and 7, and then daily until study termination at Study Day 17. The scale for clinical scoring of EAE signs of illness and/or paralysis was as follows: 0 = no disease; 1 = hind limb weakness; 2 = complete hind limb paralysis; 3 = complete hind limb and some forelimb paralysis; 4 = moribund or dead.

On day 11 post-immunization, 26 guinea pigs with a clinical EAE score of 1 were assigned to the different dose groups, and treated by s/c injection with either natalizumab, GG5/3, or sterile saline as a control, as outlined in Table 3 below, from the sponsor's final study report.

Table 3. Treatment paradigm for AN10026 or GG5/3 in EAE model in guinea pigs.

Group	Treatment	Lot#	n	Dose level (mg/kg)	Dose Volume (ml/kg)	Dose Solution (mg/ml)
A	Saline	-	9	-	1	-
B	GG5/3	583	8	3	1	3.0
C	Antegren	C0237	9	3	1	3.0

Antibody or control article injections were repeated three days later. Body weights and clinical scores were recorded daily until Study Day 17, by an evaluator who was blinded as to the treatment assignment. Prior to terminal sacrifice, blood was collected from all animals by cardiac puncture under light isoflurane anesthesia. Guinea pigs were then euthanized by CO_2 inhalation, and the brain and spinal cord isolated from each animal and flash-frozen immediately in 2-methylbutane for immunohistochemical analysis. Tissue sections were cut at 15 μ thick, incubated with MCA 518 antibody for staining of monocytes and MCA 751 antibody for T-lymphocytes, and processed for immunohistochemistry staining. All slides were reviewed microscopically to evaluate the degree of lymphocyte and monocyte infiltration into the brain and spinal cord, by an observer who was blinded as to the treatment assignment.

Results: Body weights were determined as a measure of clinical status in all animals on study, as shown in Figure 5, from the sponsor's final study report, below. Body weights in the groups of guinea pigs treated with either natalizumab or GG5/3 anti-α4 integrin monoclonal antibody were significantly different from animals in the vehicle control group on Study Days 12-17 ($p = 0.0012$, repeated-measures analysis of variance with Student-Newman-Keuls comparison). There were no statistical differences in body weights between the groups of animals treated with either GG5/3 or natalizumab.

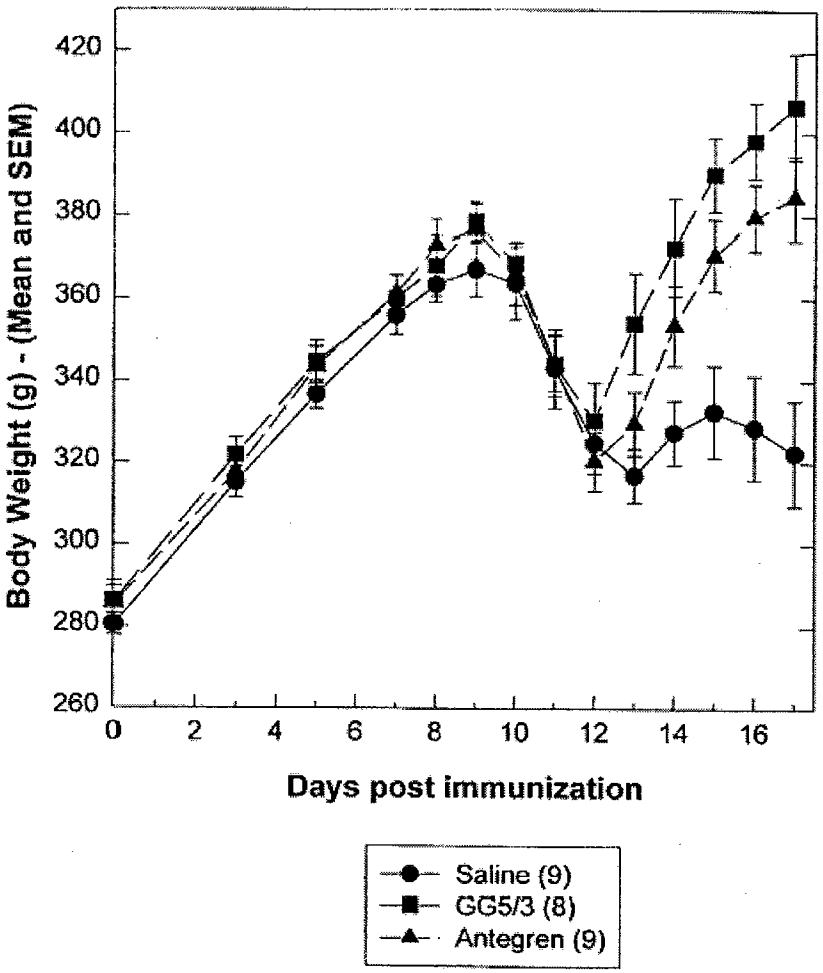


Figure 5. The effect of Antegren (natalizumab) vs. GG5/3 on body weight changes in the guinea pig EAE reversal model.

Clinical scores were also statistically significantly improved in animals treated with either AN100226 or GG5/3 at these time points (p = 0.003, Kruskall-Wallis test), as shown in Figure 6 below, from the sponsor's final study report. The number of animals with clinical scores > 1 between Study Days 11 and 17 actually improved in the two groups treated with natalizumab or GG5/3, so that at study end scores were elevated in 1/8 animals treated with GG5/3, and 3/9 animals receiving AN100226 (data not shown in this review). No statistically significant differences in clinical scores between the guinea pigs treated with AN100226, and those receiving the GG5/3 monoclonal antibody were noted at any time point on study.

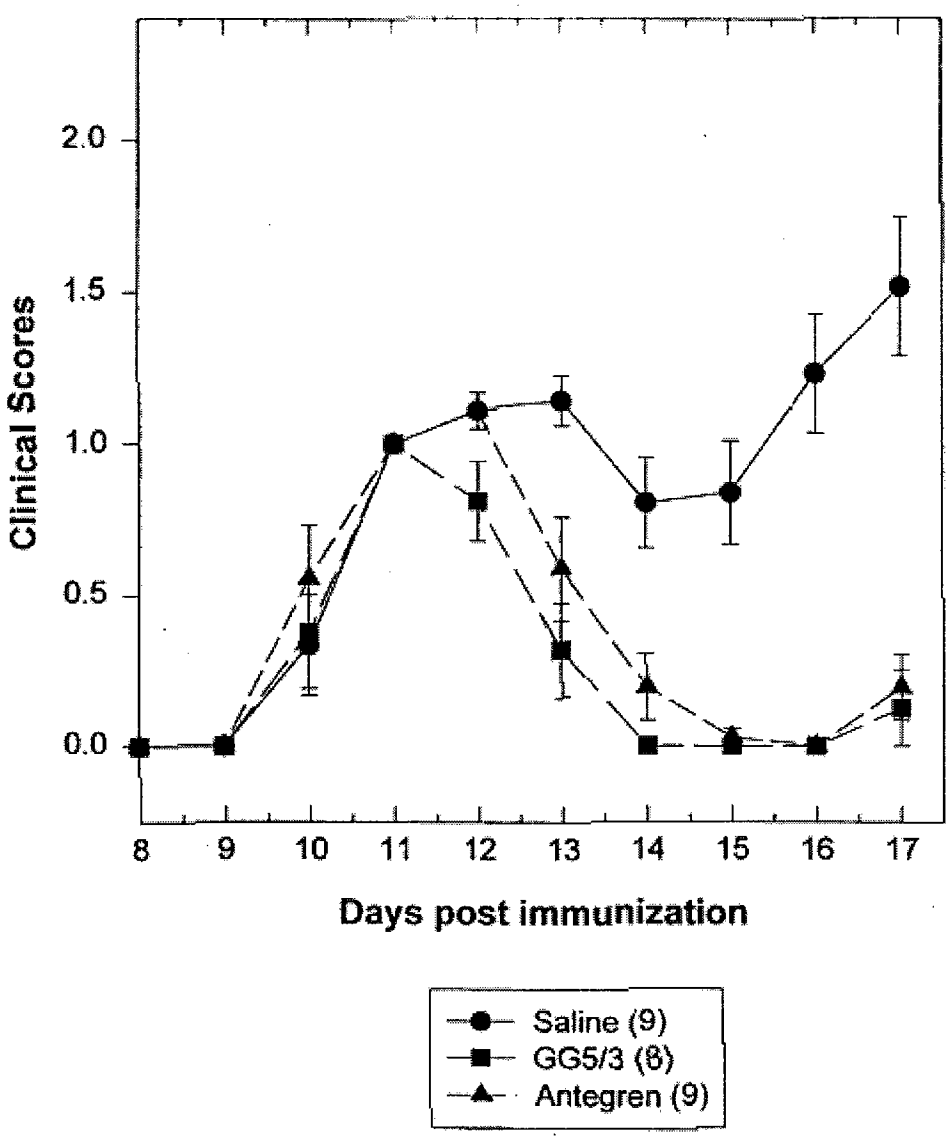


Figure 6. The pharmacologic effects of Antegren —— (natalizumab) vs. GG5/3 on clinical score changes in the guinea pig EAE reversal model.

Microscopic evaluation of immunohistochemically stained tissue sections from EAE guinea pig brain and spinal cord obtained at terminal sacrifice on Study Day 17 revealed statistically significant decreases in the numbers of both infiltrating monocytes and T lymphocytes in animals

receiving two injections of either natalizumab or GG5/3 antibody, as compared to guinea pigs treated with saline as the vehicle control ($p = 0.001$, analysis of variance with Student-Newman-Keuls comparison). Again, there were no significant differences in the infiltrative response observed for either cell subset between the AN100226 and the GG5/3 treated animals. The results of this study are shown below in Figure 7, which was provided by the sponsor in the final study report.

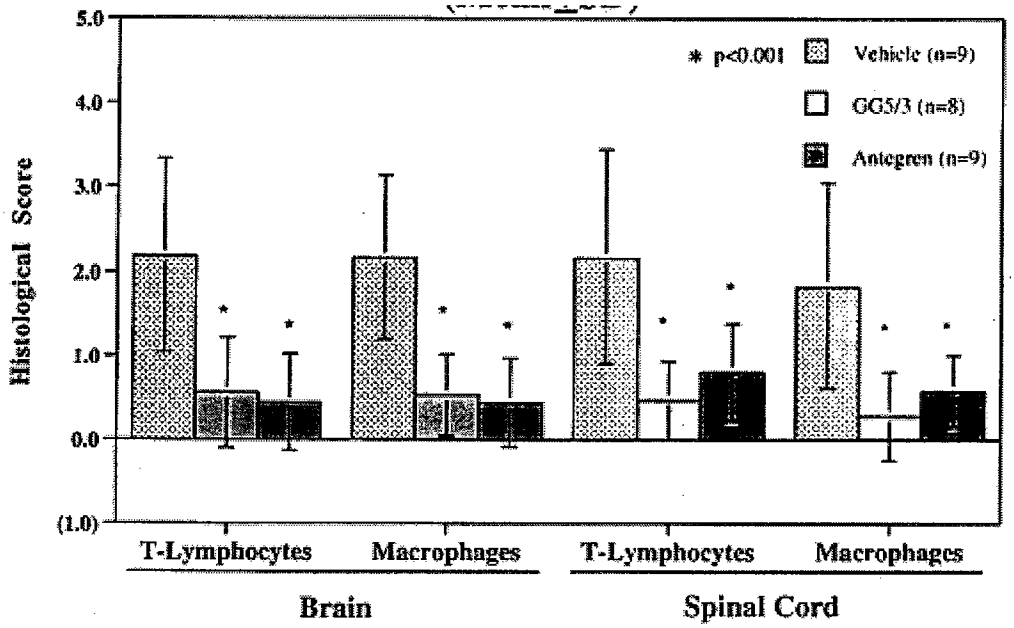


Figure 7. Effects of AN100226 or GG5/3 anti-a4 integrin antibody on infiltration of inflammatory cell subsets in EAE guinea pig brain and spinal cord.

Study conclusion: The humanized anti-α4 integrin antibody AN100226 (natalizumab) was similarly effective to the positive control antibody GG5/3 in preventing the clinical signs (body weight loss and deterioration of clinical score) in a guinea pig model of EAE. Additionally, both antibodies inhibited infiltration of T lymphocytes and monocyte/macrophages into EAE brain and spinal cord. Taken together, these data confirm that AN100226 produced by the [redacted] scale-up process is biologically active in the guinea pig EAE model, and behaves similarly to the positive control antibody GG5/3.

Study title: Efficacy of AN100226 in guinea pig experimental allergic encephalomyelitis after subcutaneous administration.

Key findings: Both natalizumab and AN100226m anti-α4 integrin monoclonal antibodies could protect guinea pigs from clinical signs of EAE-induced illness, and prevent infiltration of brain and spinal cord by inflammatory leukocytes in a dose-related manner in an experimental EAE model of MS.

Study #: AL078 (laboratory report #94124)

Volume # and page #: EDR file: STN BLA 125104\000\module4\primarypharm\al078.pdf

Conducting laboratory and location: [redacted]

Date of study initiation: not specified (final report dated May 18, 1994)
GLP compliance: no
QAU statement: yes ( ) no (X)
Drug, lot #, and % purity: AN100226 (humanized version; natalizumab), lot #7904/10, 3.0 mg/ml; AN100226m, lot #617.61, 4.14 mg/ml; both formulated in phosphate buffered saline; percent purity of either antibody preparation was not specified in the final study report

Methods: The ability of natalizumab as compared to the parent murine anti-α4 integrin antibody to protect guinea pigs from clinical signs of illness was compared in an experimental allergic encephalitis model. Three to four-week old, female Hartley strain guinea pigs (weight range 200-250 gm) were immunized by s/c injection with a suspension of homogenized guinea pig CNS tissue in Freund's complete adjuvant. In the first experiment, the time course of mononuclear cell infiltration into the brain was initially determined to start between days 6 and 8 post-immunization. Therefore, guinea pigs (n = 8/group) were treated with either vehicle (PBS), 0.03, 3, or 3 mg/kg AN100226m or natalizumab on days 7 and 10 post-immunization. Peripheral blood samples were obtained 24 h after the second injection, and analyzed for serum levels of anti-α4 integrin antibody to confirm exposure. Clinical scores were evaluated daily using the scale described in Study #310-1-A, above, and the number of days for each guinea pig to reach a clinical score of 1 was recorded. Data were analyzed for statistically significant differences between the treatment groups by ANOVA. All animals in this experiment were euthanized on Study Day 20 without any additional evaluation.

In the second experiment, the ability of AN100226m and natalizumab to reverse clinical signs of EAE illness was compared. Female Hartley guinea pigs were immunized with isogenic brain homogenate emulsified in Freund's complete adjuvant on Study Day 0, and allowed to develop clinical signs of EAE illness. On Study Day 13 following immunization guinea pigs with a clinical EAE score of 1 were randomized to the different dose groups, and treated by s/c injection with either PBS, or 0.3, 1.0, or 3.0 mg/kg natalizumab or 3.0 mg/kg AN100226m. Injections were repeated on Study Day 16. Peripheral blood samples for FACS analysis of AN100226 or AN100226m antibody levels were obtained on Study Day 14 approximately 24 hours after the initial antibody treatment, and on Study Day 19 prior to euthanasia. All animals were terminated on Study Day 19, brain and spinal cord tissue samples collected, and processed for immunohistochemical staining of infiltrating monocyte/macrophages and T lymphocytes as previously described. Histological analysis was performed by a blinded reviewer. The area under the concentration-time curve (AUC) for AN100226m or natalizumab between Study Days 13 and 19 was calculated using the trapezoidal rule. All data were analyzed for statistical significance between the treatment groups by ANOVA.

Results: In the first experiment, the effect of natalizumab and AN100226m in delaying the onset of clinically significant EAE illness was determined. In an initial pilot study, immunohistochemical staining of brain and spinal cord sections from EAE guinea pigs revealed the appearance of T lymphocytes beginning days 6 and 7 following immunization, and progressively increasing infiltrates present on days 8 and 9. By day 10 post-immunization, brain sections showed meningeal inflammation, perivascular lymphocyte cuffing, and widespread infiltration of T lymphocytes and monocytes into the brain parenchyma (data not shown). Therefore, animals were treated with AN100226m or natalizumab on Study Days 7 and 10 after immunization in all subsequent studies.

Treatment of guinea pigs immunized with isogenic CNS tissue with 3 mg/kg/dose of either AN100226m or natalizumab resulted in a statistically significant delay in onset of disease, as determined by the time to reach a clinical score of 1.0. Natalizumab and AN100226 treatment at 3 mg/kg/dose resulted in a delay in time to reach a clinical score of 1 by 4.6 and 3.0 days,

respectively, as compared to the vehicle control group. There were no significant effects of natalizumab or AN100226m on time to disease onset at the two lower dose levels, and no significant difference in time to disease onset between the groups receiving AN100226m and natalizumab at any dose level. The mean values for time to clinical score of 1.0 are shown in Table 4 below, which was derived from data included in the sponsor's final study report.

Table 4. Effect of AN100226m and natalizumab on disease onset in a guinea pig EAE model

Antibody Treatment	Mean Time to Clinical Score of 1.0 (days), ± S.D.		
	0.03 mg/kg	0.3 mg/kg	3.0 mg/kg
Vehicle (PBS)	11.0 ± 1.4		
AN100226m	10.0 ± 1.6	11.6 ± 1.4	14.0 ± 2.3[a]
Natalizumab	10.9 ± 1.5	10.9 ± 1.2	15.6 ± 1.3[b]

[a] statistically significant vs. PBS control (p = 0.007, Kruskall-Wallis test)

[b] statistically significant vs. PBS control (p = 0.000, Kruskall-Wallis test)

A similar protection of guinea pigs from the EAE-associated loss in body weight was also observed for the two groups of animals treated with 3.0 mg/kg/dose anti-α4 integrin antibody. Guinea pigs treated with either vehicle or 0.03 or 0.3 mg/kg/dose of either antibody showed decreased body weights from baseline, beginning on Study Day 10 prior to the onset of clinical disease. By contrast, animals receiving 3.0 mg/kg of AN100226m or natalizumab gained weight over the initial portion of the study, and did not start to lose body weight until EAE disease was clinically evident, beginning on Study Day 15. The results are shown in Figure 8 below, which was provided by the sponsor in the final study report.

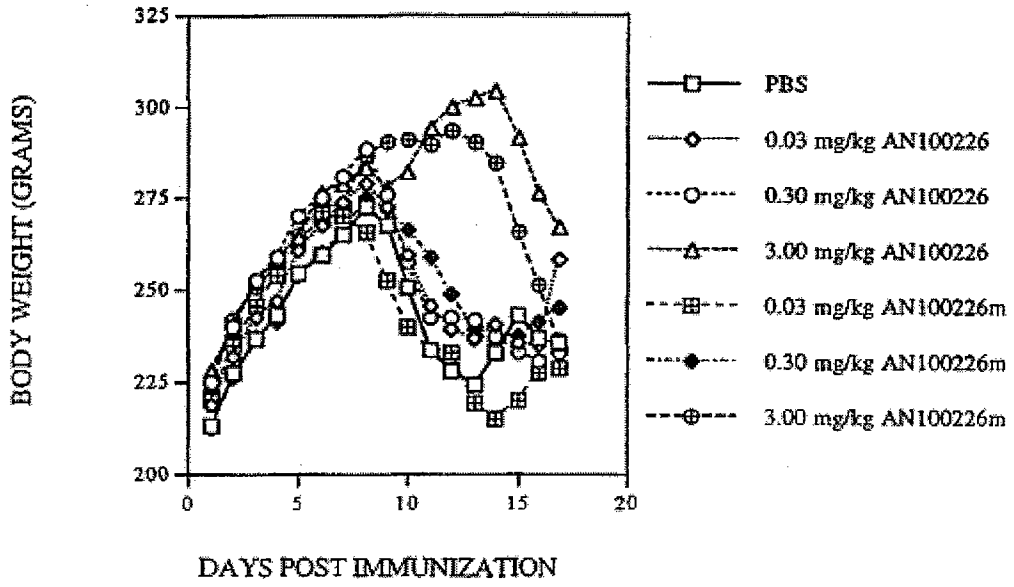


Figure 8. Body weight measurements for EAE guinea pigs treated s/c with vehicle (PBS), AN100226m, or natalizumab on Study Days 7 and 10.

Serum levels of the anti-α4 integrin antibodies were measured in samples obtained from 3 pigs/group on Study Day 11, by flow cytometric evaluation of their binding to freshly isolated guinea pig lymphocytes from naïve animals. Mean fluorescent intensities for AN100226m and natalizumab titers in serum from treated animals were quantitated from the linear portion of a standard curve generated with known amounts of the respective antibody present. Serum AN100226m and natalizumab levels from guinea pigs treated with 0.03 or 0.3 mg/kg/dose of the respective antibodies were not detectable in this assay (data not shown). Following s/c dosing with 3.0 mg/kg/dose of natalizumab or AN100226m, mean serum levels were 21.8 ± 1.8 μg/ml and 18.7 ± 4.8 μg/ml for the two antibodies, respectively.

In the second experiment, the ability of the anti-α4 integrin antibodies to reverse established EAE clinical illness was evaluated. Guinea pigs with clinical EAE scores of 1.0 on Study Day 13 received two injections of either 0.3, 1.0, or 3.0 mg/kg/dose natalizumab, or 3.0 mg/kg/dose AN100226m. Clinical scores in established EAE were markedly improved beginning on Study Day 16 in both groups treated with 3.0 mg/kg/dose of the anti-α4 integrin antibody; however, the response was statistically significantly different from the PBS control group only in the animals treated with 3 mg/kg/dose of the humanized AN100226 molecule ($p = 0.042$, ANOVA). By Study Days 17 and 18 at 25 and 48 hours after the second dose of natalizumab, all 8 guinea pigs in this treatment group had clinical scores of 0, and were considered disease-free. The results of this study are presented in Figure 9 below, which was obtained from the sponsor's final study report.

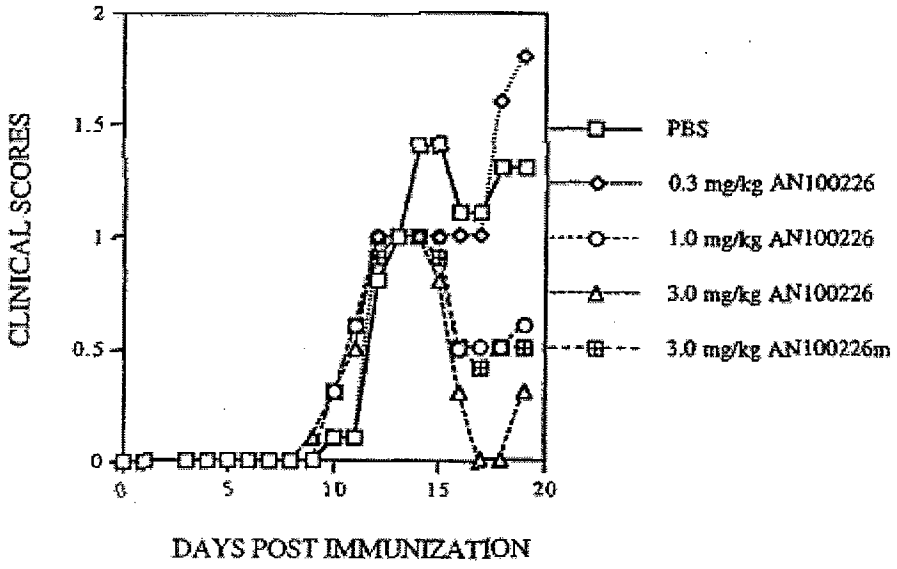


Figure 9. Effect of AN100226m and natalizumab (AN100226) treatment on reversal of EAE-associate clinical scores in guinea pigs.

Similar protective effects of natalizumab or AN100226 antibody treatment were observed on EAE-related body weight loss. Guinea pigs treated with 3.0 mg/kg/dose of either antibody progressively gained weight, starting within 24 hours after the first antibody dose and continuing until study termination at Study Day 19. These results were significantly different from the PBS control group only in the animals treated with 3 mg/kg/dose of the humanized AN100226

molecule (p = 0.042, ANOVA). The results are presented in Figure 10 below, which was provided in the sponsor's final study report.

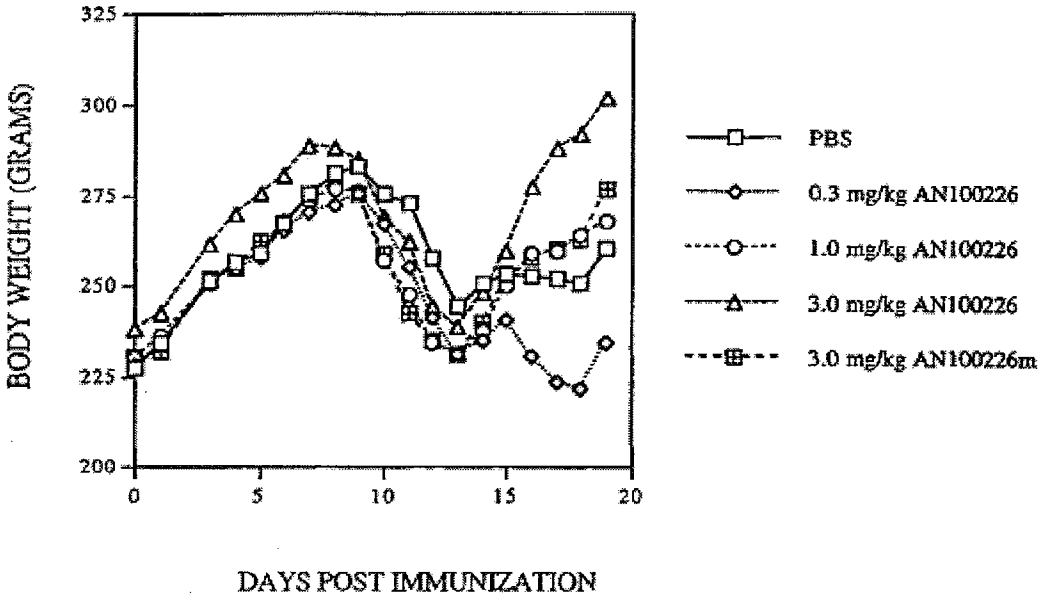


Figure 10. Effects of AN100226m and natalizumab (AN100226) on body weights of EAE guinea pigs.

Serum anti-α4 integrin antibody levels were measured 24 hours after initial treatment on Study Day 14, and at termination on Study Day 19, approximately 72 hours following the final dose. On Study Day 14, the serum levels of AN100226m treated guinea pigs were slightly lower than those obtained for the group of animals treated with 3.0 mg/kg/dose natalizumab (9.1 ± 0.9 vs. 12.6 ± 3.8 µg/ml, respectively). At termination on Study Day 19, AN100226m levels were almost undetectable (0.5 ± 0.6 µg/ml), while serum concentrations of natalizumab had decreased to approximately 6.1 ± 4.7 µg/ml, which is below the concentration of 10 µg/ml needed for receptor saturation.

Microscopic evaluation of sections of brain and spinal cord from natalizumab and AN100226m treated, EAE guinea pigs demonstrated a dose-related decrease in infiltrating T lymphocytes and monocyte/macrophages. These results are shown in Table 5, below, which was copied directly from the final report for this study. Statistically significant decreases in leukocyte infiltration into the brain, and in T lymphocyte migration into spinal cord were noted only in the groups treated with 3.0 mg/kg of either anti-α4 integrin antibody, as compared to animals treated with PBS control. There were no statistically significant differences in macrophage infiltration into the spinal cord at any dose of natalizumab tested.

Table 5. Effects of natalizumab (AN100226) and AN100226m on leukocyte infiltration into brain parenchyma and spinal cord of EAE rats.

	BRAIN		SPINAL CORD	
Group	T-cells	Macrophages	T-cells	Macrophages
PBS	2.6±0.5	2.7±0.5	2.0±0.8	2.1±0.7
AN100226				
3.0 mg/kg	0.6±0.2***	1.5±0.6**	0.9±0.2*	1.7±0.5
1.0 mg/kg	1.6±0.8	1.9±0.8	1.5±0.5	2.0±0.8
0.3 mg/kg	2.2±0.4	2.5±0.5	1.7±0.5	2.2±0.4
AN100226m				
3.0 mg/kg	0.8±0.4***	1.9±0.4**	0.9±0.2*	1.9±0.4

* p = 0.008, Kruskall-Wallis non-parametric analysis

** p = 0.005, Kruskall-Wallis non-parametric analysis

*** p = 0.001, Kruskall-Wallis non-parametric analysis

Study conclusion: The humanized anti-α4 integrin antibody AN100226 (natalizumab), and the murine parent monoclonal antibody AN100226m directed against α4 integrin were similarly effective in delaying the onset of clinical signs of EAE illness in guinea pigs, and in reversing established disease. These effects included improvements in clinical status, body weights, and a decrease in the infiltration of T lymphocytes and monocyte/macrophages into EAE brain and spinal cord. Taken together, these data confirm that the humanized AN100226 (natalizumab) antibody is comparable in effect to the murine parent monoclonal antibody AN100226m.

Study title: A monoclonal antibody to α4 integrin suppresses and reverses active experimental allergic encephalomyelitis.

Key findings: Treatment of guinea pigs with 1.5 mg/dose (approximately 6.0 to 7.5 mg/kg/dose) of the murine monoclonal antibody AN100226m directed against α4 integrin early after induction of experimental allergic encephalomyelitis (EAE) significantly delayed onset of the clinical signs of EAE-induced illness, and prevented infiltration of brain and spinal cord by inflammatory leukocytes. Treatment with AN100226m after the onset of EAE disease could also reverse the clinical signs, and resulted in clearance of activated leukocytes from the central nervous system.

Study #: none assigned (provided as a publication from the literature; Kent, S.J., S.J. Karlik, C. Cannon, D.K. Hines, T.A. Yednock, L.C. Fritz, and H.C. Horner. *J. Neuroimmunol.*, **58**:1-10, 1995)

Volume # and page #: EDR file: STN BLA 125104\000\module4\primarypharm\pb554.pdf
Conducting laboratory and location: Athena Neurosciences, 800 Gateway Boulevard, South San Francisco, CA 94080, and Department of Diagnostic Radiology and Nuclear Medicine and Physiology, University of Western Ontario, 339 Windermere Road, London, Ontario, Canada N6A 5A5
Date of study initiation: not specified
GLP compliance: no

QAU statement: yes ( ) no (X)
Drug, lot #, and % purity: AN100226m, lot number not specified, 8.4 mg/ml; percent purity of the antibody preparation was not specified in the published report

Methods: The parent murine anti-α4 integrin antibody AN100226m was evaluated for its ability to protect guinea pigs from clinical signs of illness in an experimental allergic encephalitis model. Female Hartley strain guinea pigs (weight range 200-250 gm) were immunized by i/d injection into the nuchal region with a suspension of homogenized guinea pig CNS tissue in Freund's complete adjuvant. In this model of EAE, 98% of the immunized guinea pigs begin to show acute signs of clinical illness approximately 9 days post-immunization, and continue into a chronic progressive phase starting day 21-25 post-immunization. Animals were assessed daily for clinical signs of illness, using a 4-point scale as described, below. A score of 0 was assigned if no abnormalities were present; a score of 0.5 for more than 1 day of weight loss; a score of 1.0 was assigned for hindlimb weakness and/or poor righting reflex; a score of 2.0 was given for evidence of paresis, urinary incontinence, or and/or fecal impaction; a score of 3.0 was assigned for animals with evidence of paralysis, and a score of 4.0 was assigned for guinea pigs with terminal paralysis.

In the first series of experiments to determine whether AN100226m could delay the onset of EAE clinical disease, guinea pigs (n = 9/group) received intracardiac injections on Study Days 5 and 9 post-immunization of 1.5 mg/animal AN100226m, the isotype-matched murine IgG_1 control antibody 1G5, or an equivalent volume of sterile PBS. A second cohort of 6 guinea pigs/dose group received intracardiac injections of 1.5 mg/dose AN100226m, 1G5, or the anti-α4 integrin monoclonal antibody L25 on Study Days 5, 9, 12, and 15. For the second series of experiments to determine if AN100226m could reverse clinically evident EAE, guinea pigs immunized with isogenic CNS homogenate and Freund's complete adjuvant were allowed to develop clinical signs of disease (as defined by a clinical score $\geq$ 1.0) prior to treatment on days 12 and 15 with 1.5 mg/animal of AN100226m (n = 18), 1G5 control antibody (n = 10), or PBS (n = 8). All experiments were terminated at 42 days post-immunization, at which time the animals were euthanized, and the brain and spinal cord tissues dissected for immunohistochemical and histopathological evaluation. Tissue sections for immunohistochemical staining of T lymphocyte and monocyte/macrophage subsets were snap frozen in liquid nitrogen, sectioned at 12 μ, and labeled with either MCA518 monoclonal antibody directed against macrophages, or MCA751 antibody against T cells. Antibody binding was detected using a commercially available avidin-biotin-horseradish peroxidase conjugate test kit, developed with diaminobenzidine in the presence of hydrogen peroxide, and counterstained with hematoxylin. For each animal, 3 sections each of brain and spinal cord were examined microscopically and scored for infiltrating cells using the following scale:

0	=	no infiltrating cells
+	=	very few cells, mainly perivascular
++	=	few vessels with leukocyte "cuffing," few cells radiating parenchymally
+++	=	many vessels with "cuffing" present, leukocytes invading throughout the parenchyma

Additional CNS tissue samples from each animal were fixed in 10% buffered formalin, processed through paraffin and sectioned, and stained with hematoxylin and eosin or Solochrome-R-cyanin for microscopic evaluation of inflammatory response and demyelination. Six sections per animal from each of two brain and two spinal cord locations were examined microscopically for evidence of inflammatory disease. The severity of the findings was graded by the reviewing

pathologist using a scale of 0-4 for each of the following parameters, meningeal inflammation, perivascular inflammation, encephalitis, and demyelination. The scores for each of the 4 CNS locations were combined to give a cumulative rating between 0 and 16 for each pathological feature.

Peripheral blood samples were obtained in both series of experiments to determine the effects of AN100226m treatment on total and differential leukocyte counts, and serum AN100226m and anti-AN100226m antibody concentrations. Serum levels of AN100226m were quantitated by flow cytometry as previously described in Study #AL078, using human Jurkat T lymphocytes as the target α4-integrin expressing cells, and comparing the results from the animal sera to a standard curve generated with known concentrations of AN100226m used for binding. Antibodies to AN100226m were detected using a sandwich ELISA technique, with AN100226m-coated plates as the capture antigen. Diluted serum samples from the test animals were added to the wells, incubated and washed, and exposed to [redacted] After washing, the plates were developed with ortho-phenylenediamine as the chromogen, in the presence of hydrogen peroxide, and read for absorbance using a 96-well microtiter plate reader.

Results: In the first experiment, the effects of AN100226m in delaying onset of clinically significant EAE illness were determined. Clinical signs of disease were first evident in control animals on Study Day 9 following immunization, followed by a rapid increase in clinical illness that peaked on Study Day 15 with a mean clinical score of 1.0 ± 0.22 for animals treated with PBS. A similar course of disease was observed in the guinea pigs treated with 1.5 mg/dose 1G5 control antibody on Study Days 5 and 9. By contrast, clinical signs of EAE disease were significantly delayed in the group of animals treated on Study Days 5 and 9 with 1.5 mg/dose AN100226m, with the onset of disease first noted at Study Day 12 post-immunization. The results of this study are shown in Figure 11 panel A, which was copied directly from the sponsor's publication submitted to the BLA.

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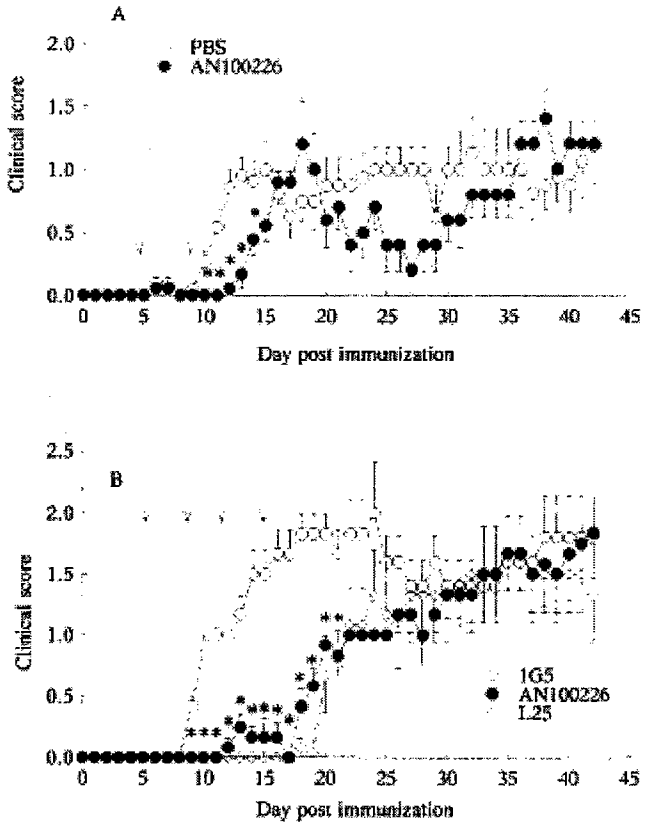

Figure 11. Inhibition of onset of clinically detectable EAE disease by anti-α4 integrin monoclonal antibodies. Panel A, clinical course of disease development in EAE guinea pigs treated on Study Days 5 and 9 with PBS (open circles), or AN100226m (solid circles). Panel B, clinical course of disease in guinea pigs treated with 1G5 control antibody (open circles), AN100226m (solid circles), or the positive control anti-α4 integrin antibody L25 (open diamonds) on Study Days 5, 9, 12, and 15. An asterisk indicates a significant difference between the control and the AN100226m treated groups ($p \leq 0.05$, Mann-Whitney rank sum test).

A similar delay in onset of disease was observed in guinea pigs treated with AN100226m, 1G5, or L25 anti-α4 integrin antibody as a positive control on Study Days 5, 9, 12, and 15. These results are shown in Figure 11, panel B (above), from the final study publication. Repeat administration of either L25 or AN100226m antibodies resulted in statistically significant delays in clinical illness ($p < 0.05$, Mann-Whitney rank sum). However, in both cases, once treatment with the anti-α4 integrin antibodies was stopped, all animals developed progressive disease between Study Days 25 and 30, and continued to worsen until study termination on day 42 post-immunization.

Histopathological and immunohistochemical analysis of sections of brain and spinal cord from EAE guinea pigs treated with AN100226m in either series of experiments showed marked reductions in the infiltration of both T lymphocytes and monocytes, as compared to the levels observed in either the PBS or 1G5 control groups. The results of these examinations are presented in Table 6 below, which was provided in the sponsor's publication. Although AN100226m could inhibit leukocyte infiltration into brain and spinal cord tissues, there were no

effects of antibody treatment on either total or differential peripheral blood leukocyte counts (data not shown).

Table 6. Histopathological and immunohistochemical analysis of CNS tissues from EAE guinea pigs treated with PBS, 1G5 control antibody, or AN100226m.

Animal (sac)	Treatment	T cells	Monocytes	Path score (MPED) [a]
S001 (day 13)	PBS day 5, day 9	+ + +	+ + +	[b]
S008 (day 13)		+ + +	+ + +	[b]
S010 (day 13)		+ + +	+ + +	[b]
S013 (day 13)	AN100226m day 5, day 9	+	0	[b]
S014 (day 13)		+	0	[b]
S018 (day 13)		+	0	[b]
S020 (day 13)		+	0	[b]
S311 (day 14)	PBS day 12, day 15	+ + +	+ + +	7,13,8,7
S320 (day 17)		+ + +	+ + +	9,10,8,6
S511 (day 13)	1G5 day 12, day 15	+	+ + +	7,10,6,5
S519 (day 14)		+ + +	+ + +	2,6,5,2
S512 (day 16)		+ +	+ + +	5,10,8,8
S502 (day 18)		+	+ + +	6,9,5,7
S510 (day 13)	AN100226m day 12, day 15	+	+ + +	1,0,0,0
S516 (day 14)		0	+ +	1,0,0,0
S518 (day 16)		0	+	2,3,3,3
S318 (day 17)		+	+ +	1,1,1,0
S301 (day 17)		0	+ +	0,0,0,0
S521 (day 18)		0	+	0,0,0,0

Animals were immunized on day 0 to initiate EAE and were injected with PBS, AN100226m or 1G5 on the days indicated. T cell and monocyte infiltration was determined by standard immunocytochemistry using the ABC method. The pathological score was determined from formalin-fixed sections stained with Hematoxylin–Eosin or Solochrome R-cyanin (Karlik et al., 1986). Each of the numbers corresponds to a cumulative score from four CNS locations (two brains, two spinal cords) in one of four categories.

[a] M, meningeal inflammation; P, perivascular inflammation; E, encephalitis; and D, demyelination.

[b] Tissue was sampled for immunocytochemical analysis only.

Serum concentrations of AN100226m, as determined by flow cytometric analysis of binding to human Jurkat T cells were at or below the limit of the assay detection (20 ng/ml) on Study Day 12 in the animals receiving treatment on days 5 and 9, suggesting that the antibody is rapidly cleared from the circulation by 72 hours after injection. By contrast, low although detectable serum levels of AN100226m were present at Study Day 13 following injections of 1.5 mg/dose on Study Days 5, 9, and 12. The values for the three animals sampled at this time point were 26, 14, and 22 µg/ml (mean 20.7 $\pm$ 6.1 µg/ml) AN100226m. Antibody titers against AN100226m were also detectable in both of these cohorts. However, titers in all three guinea pigs treated with AN100226m on Study Days 5 and 9 were > 1:1250, while animals receiving injections on Study Days 5, 9, and 12 had anti-AN100226m antibody titers of 1:250, 1:250, and 1:50, as determined by ELISA.

Comment: There are no data included in the published study report that demonstrate the assay sensitivity of the sandwich ELISA used to detect anti-AN100226m antibody following treatment of EAE guinea pigs. Specifically, it cannot be determined from the information provided whether significant levels of AN100226m present in serum can interfere with the ELISA assay for detection of immunogenicity. Although the low serum levels of AN100226m coupled with the seemingly high titer of anti-AN100226m antibody in animals treated on Study Days 5 and 9 suggest that the host antibody response is rapidly eliminating the mouse monoclonal protein, detectable AN100226m was present on Study Day 13, approximately 24 hours after a third injection was given, and the anti-AN100226m antibody titers appear to be much lower. These results may be due to a failure of the assay to detect significant anti-AN100226m antibodies in the presence of relatively high concentrations of the product.

The second series of experiments evaluated the ability of AN100226m to reverse the hindlimb weakness and/or paralysis associated with acute EAE disease. For these studies, animals were immunized to induce EAE on Study Day 0, and allowed to progress until clinical scores of at

least 1.0 were reported. Clinical signs of illness became evident starting on Study Day 9, and by Study Day 12 all animals had a clinical score of at least 1.0, at which point treatment with either PBS, or 1.5 mg/dose AN100226m was initiated. The control or test article injections were repeated on Study Day 15, and guinea pigs were evaluated daily for clinical signs of EAE disease until termination. In this study, selected animals were euthanized between Study Days 13 and 18, and brain and spinal cord samples were processed and stained for histopathological and immunohistochemical evaluation of infiltrating leukocytes and CNS pathology. All other animals were observed until Study Day 42 post-immunization.

Administration of 1.5 mg/dose AN100226m on Study Days 12 and 15 post-immunization was able to statistically significantly reverse the clinical EAE scores, as compared to the PBS or 1G5 control groups ($p \leq 0.05$, Mann-Whitney rank sum test). However, these effects were transient, and animals began to progressively worsen beginning on approximately Study Day 20, or 5 days after the final dose of AN100226m. The results of this experiment are shown in Figure 12 below, from the sponsor's published study report.

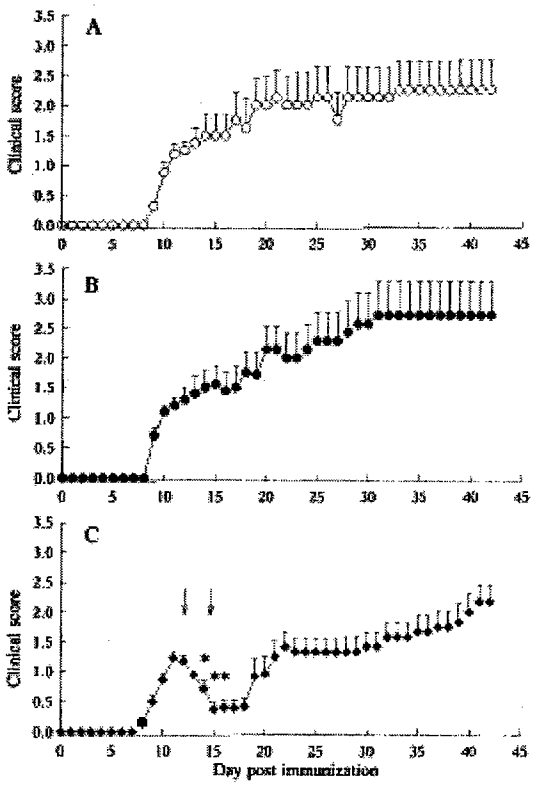

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Figure 12. Reversal of EAE clinical scores by treatment of guinea pigs with anti-α4 integrin monoclonal antibody AN100226m on Study Days 12 and 15. Panel A, PBS control. Panel B, 1G5 mouse IgG_1 isotype-matched control antibody, 1.5 mg/dose. Panel C, 1.5 mg/dose AN100226m. Arrows indicate the day of treatment, and asterisks indicate significant differences from control animals ($p \leq 0.05$, Mann-Whitney rank sum test).

Histopathological evaluated of brain and spinal cord samples obtained from PBS or 1G5 control animals in this portion of the study showed marked infiltration of both monocytes/macrophages and T lymphocytes, and notable perivascular cuffing between Study Days 13 and 18 post-immunization. Demyelinization of both brain and spinal cord tissue was also present in samples from animals in both control groups at these time points. By contrast, a marked reversal of T lymphocyte accumulation, and a decrease in monocytes infiltration in both brain and spinal cord

2 = paresis

3 = paralysis

4 = terminal paralysis and death

Eight animals per group were evaluated by magnetic resonance imaging (MRI) for evidence of abnormalities associated with EAE disease. MR imaging was performed on a scanner, using a custom-designed coil. Fast spin-echo (T2-weighted) and T1-weighted, pre- and post-contrast scans were obtained on Study Days 12, 15, 19 (approximately 24 hours following the final dose of AN100226m), and on Study Day 25. Six coronal, non-contigual slices 3 mm wide with a 0.5 mm gap were obtained for each fast spin-echo (T2-weighted) reading. T2-weighted MR abnormalities were scored by a blinded evaluator using a published, 5 point scale in which 0 indicates no abnormality, and 4 indicates severe abnormality.[1] Pre- and post-contrast, T1-weighted scans were performed following administration of Magnevist contrast imaging agent, at a dose of 1 mmol/kg. The T1-weighted images were subjected to image analysis, and frequency histograms of pixel intensities generated and digitized, and the level of CNS contrast enhancement quantitated. Contrast data from T1-weighted scans from non-EAE guinea pigs were used to calculate the range of intensities for normal guinea pig brain, and the mean value plus 2 SD was used as the upper limit of intensity for normal brain tissue. The pixel histograms from EAE animals were compared with this value, and all pixels exceeding this defined upper limit were used to calculate the percent of pixels due to blood-brain barrier leakage of contrast material.

To more fully evaluate the effects of AN100226m on changes in blood-brain barrier permeability in the EAE model, a second group of EAE guinea pigs received daily MRI scans with and without contrast, beginning on Study Day 9 after immunization. Treatment with PBS (vehicle control) or AN100226m was initiated at a dose of 5 mg/kg, s/c on the first day that contrast enhancement was observed, then a second dose of AN100226m or vehicle was administered 3 days later. Animals in this portion of the study received daily MRI scans, and a quantitative analysis of the T1-weighted images (percentage of pixels with enhancement, post-contrast as described above) was performed.

Results: All animals developed clinical signs of acute EAE illness beginning approximately 9 days after immunization, as previously described (please see Study #AL078, above). Treatment with 1.5 mg/dose AN100226m s/c on Study Days 12, 15, and 18 could effectively reduce the clinical signs of active EAE, as shown by a statistically significant reduction in clinical scores between Study Days 18 and 21 post-immunization. These data are shown in Figure 13 below, which was obtained from the final, published study report.

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[1] Karlik, S.J. E.A. Grant, D. Lee, and J.H. Noseworthy. 1993. Gadolinium enhancement in acute and chronic-progressive experimental allergic encephalomyelitis in the guinea pig. *Magn. Reson. Med.*, **30**:326-331.

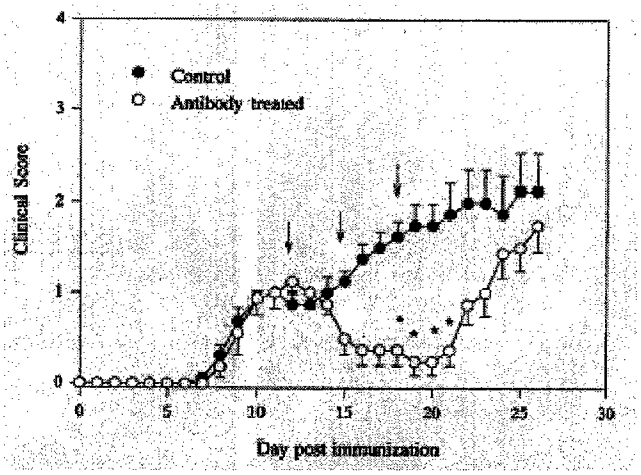

Figure 13. Reversal of clinical signs of active EAE by AN100226m treatment. EAE guinea pigs (n = 8/group) were treated with vehicle control (closed circles) or 1.5 g/dose AN100226m (open circles). Results are expressed as the mean clinical score, ± S.E. for each group. Arrows indicate the day of treatment; asterisks indicate a significant difference between the groups ($p \leq 0.05$, Mann-Whitney rank sum test).

T2-weighted, fast spin-echo MRI scans showed abnormalities consistent with areas of cerebral edema and inflammation in EAE guinea pigs at Study Day 12 after immunization, which progressively worsened at Study Days 15 and 19 in the vehicle control group. These data are shown in Figure 14, panels a, b, and c from the sponsor's publication, below. There were no remarkable differences in either the degree of cerebral edema detected, or the quantitative MR scores obtained between animals in the control and AN100226m groups prior to initiation of treatment on Study Day 12 (Figure 14, panels a and d, and Figure 15, below).

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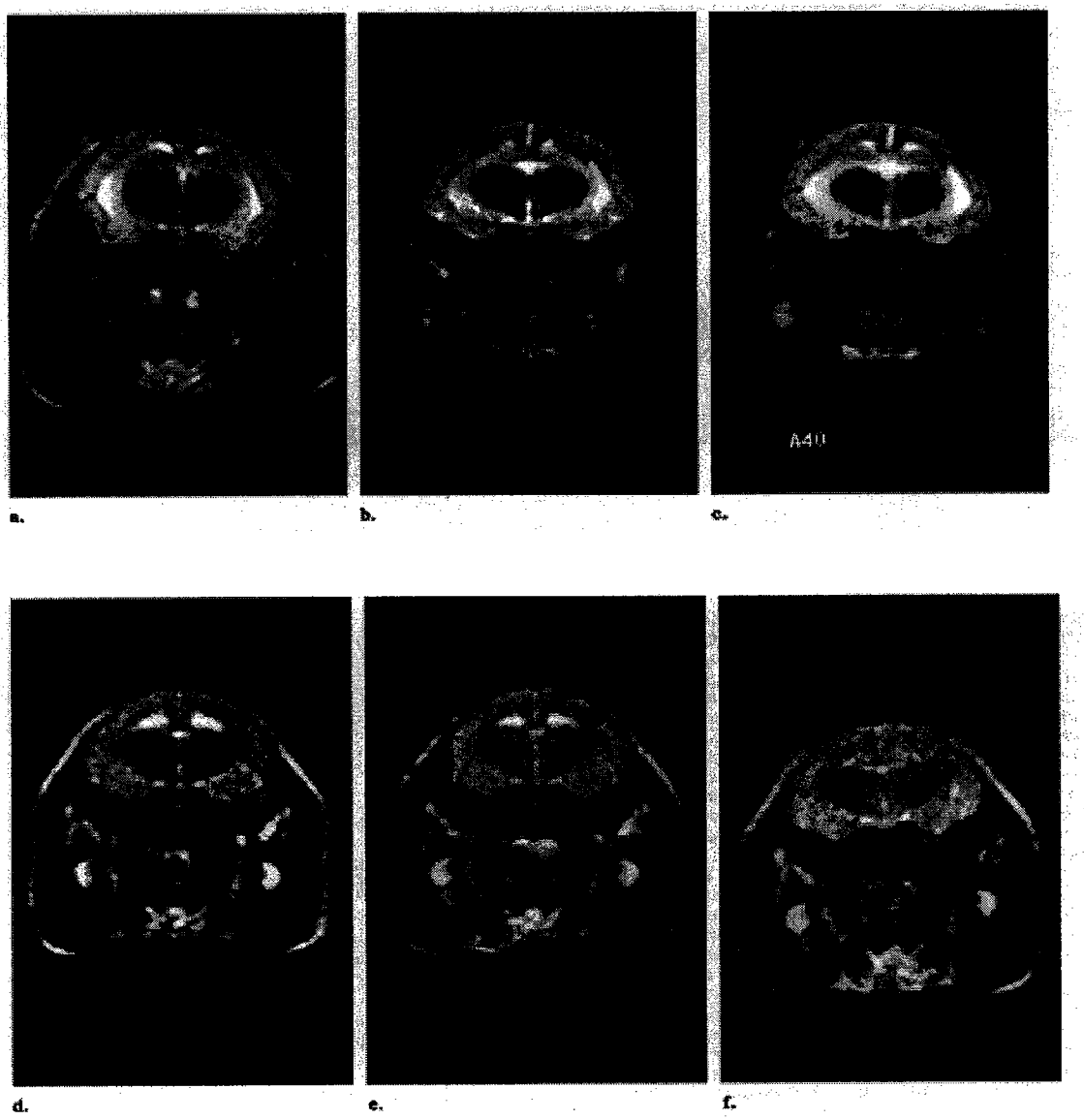

Figure 14. MRI images of cerebral edema in EAE guinea pigs following treatment with AN100226m. Panels a, b, and c are from animals treated with vehicle control; images were obtained on Study Days 12, 15, and 19, respectively. The images in panels d, e, and f were obtained at the same respective time points, from guinea pigs treated with 1.5 mg/dose AN100226m on Study Days 12, 15, and 18. Arrows indicate regions of CNS edema.

EAE guinea pigs treated with 1.5 mg/dose AN100226m on Study Days 12, 15, and 18 showed marked decreases in the abnormalities seen on T-2 weighted MR imaging (Figure 14, panels d, e, and f, above), as compared to the control animals at these same time points. Corresponding decreases in the T2-weighted MR scores were also observed in the AN100226m group, as compared to the EAE animals treated with vehicle control at Study Days 12, 15, and 18. One week following discontinuation of dosing with AN100226m, the T2-weighted MRI findings and MR score had returned to the pre-treatment levels, and were not significantly different from those obtained for animals treated with the vehicle control agent. The results of the MR scores for

cerebral edema in these animals are presented in Figure 15 below, which was provided by the sponsor in the final, published report.

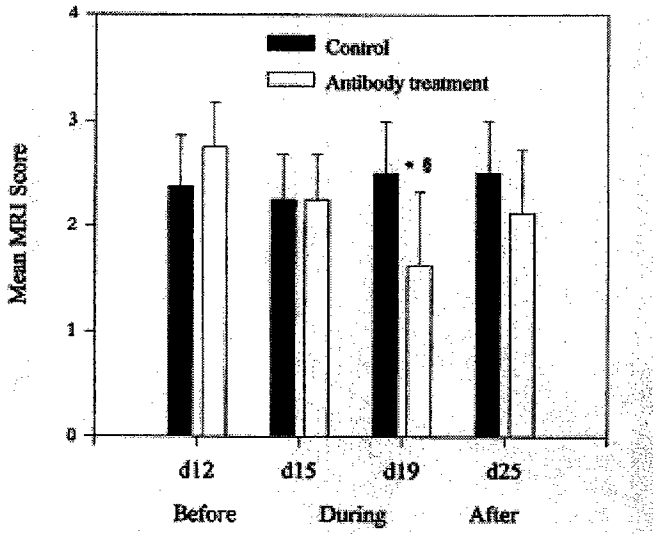

Figure 15. Mean MR imaging scores for cerebral edema following AN100226m treatment. EAE guinea pigs (n = 8/group) were treated with vehicle control (open bars) or 1.5 g/dose AN100226m (filled bars) as described in Methods. Results are expressed as the mean MR imaging score for T2-weighted scans based on a scale of 0-4, ± S.E. for each group. Asterisk indicates a significant difference between the group scores at that time point, & indicated a significant difference from the initial MR score for the AN100226m group ($p \leq 0.05$, Mann-Whitney rank sum test).

Contrast-enhanced, T1-weighted MR images also demonstrated similar improvements following AN100226m treatment. Figure 16, from the sponsor's published study report shows the degree of leakage of contrast material at the blood-brain barrier in guinea pigs with EAE. Prior to treatment, all animals had evidence of contrast enhancement in the brain parenchyma, indicating leakage (Figure 16, panels a, and b). During the treatment period (*i.e.* 4 days after initiation of treatment), control guinea pigs continued to demonstrate significant levels of contrast enhancement on MR imaging, while enhancement was markedly decreased in animals receiving AN100226m (Figure 16, panels c, and d, respectively).

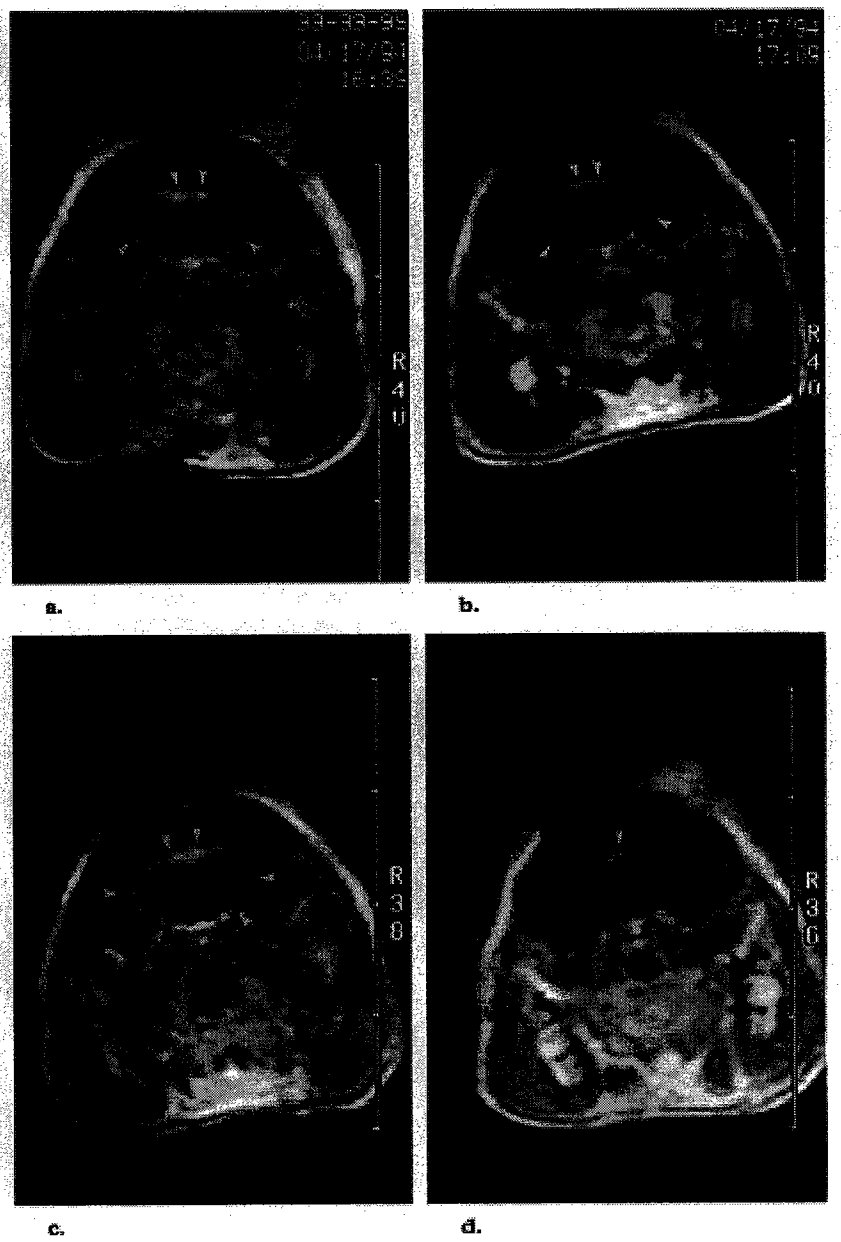

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Figure 16. Contrast-enhanced, T1-weighted MR imaging of EAE guinea pig brain. T1-weighted, post-contrast images were obtained from EAE animals on the first detectable day of contrast enhancement, prior to initiation of treatment (panels a and b), or 4 days after initiation of treatment with either vehicle control (panel c), or 1.5 mg/dose AN100226m. Arrowheads indicate regions of enhancement.

A quantitative analysis of the T1-weighted images was conducted to obtain a measure of blood-brain barrier permeability. The data are shown in Figure 17 below, as provided by the sponsor in the final, published study report.

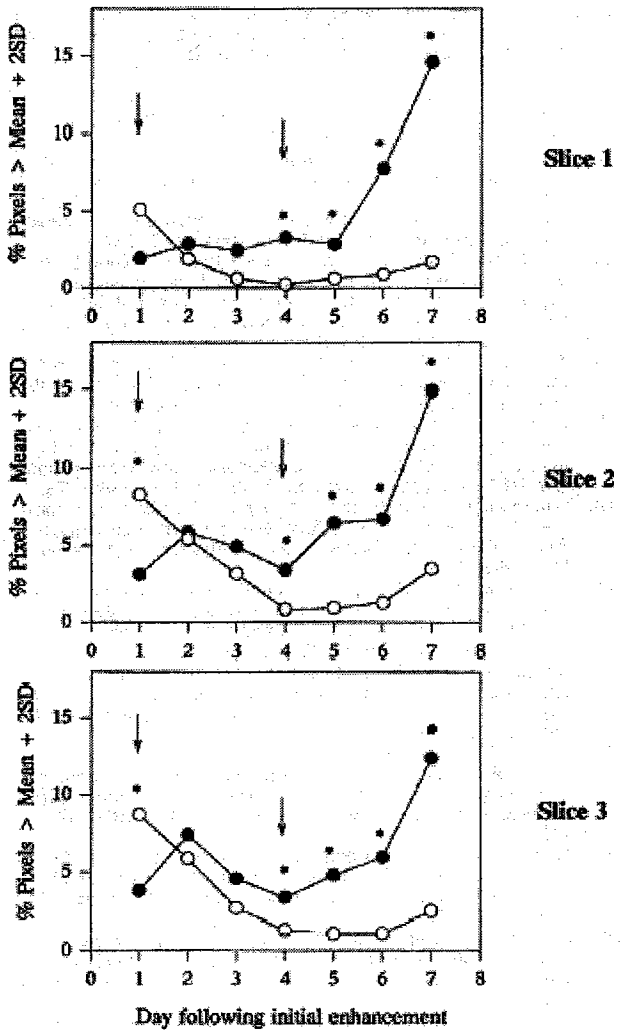

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Figure 17. Reversal of blood-brain barrier permeability by AN100226m treatment of EAE guinea pigs. Arrows indicate days of treatment with either 5 mg/kg AN100226m, s/c (open circles), or vehicle control (close circles). Asterisks indicate significant differences between the groups at that time point ($p \leq 0.05$, Mann-Whitney rank sum test).

The percentage of high-intensity pixels related to blood-brain barrier leakage of the contrast agent in EAE guinea pigs in the control group increased over the duration of the study. However, decreases in the percentage of high-intensity pixels were observed in the AN100226m treated EAE guinea pigs beginning approximately 24 h after the first dose, and were statistically significantly lower than the vehicle control group by day 4 through the remainder of the dosing period. This rapid, and sustained decrease in the percentage of high-intensity pixels related to leakage of contrast agent suggests that the increase in blood-brain barrier permeability associated with EAE disease can be reversed by AN100226m, and correlates with the reversal of the clinical signs of disease observed (Figure 13, above).

Study Conclusion: Treatment of EAE guinea pigs with AN100226m could successfully reverse the disease-related cerebral edema and increased blood-brain barrier permeability, as detected by MR imaging. Resolution of MR lesions occurred at approximately the same time points that the clinical scores for EAE illness improved. The improvements in MR scans and clinical scores were both reversed following discontinuation of AN100226m treatment. Taken

together, these data suggest that the clinical improvements noted are after AN100226m treatment are related to reversal of the blood-brain barrier permeability changes in EAE disease, likely through interference of the interaction between circulating leukocytes and the endothelial cells of the blood-brain barrier.

Study title: Characterization of AN100226 and AN100226m as inhibitory antibodies against human alpha-4 integrin.

Key findings: The murine parental anti-α4 integrin monoclonal antibody AN100226m could effectively inhibit binding of human Jurkat or Ramos T lymphocyte cells to TNF-α activated HUVEC and rat brain endothelial cells, and to the murine L-cell fibroblast cell line transfected with human VCAM-1. Binding of the human monocytic cell line U937 to inflamed endothelium from a rat brain EAE model was also completely inhibited by AN100226m. Interaction of AN100226m with human lymphocytes and monocytes, but not neutrophils was demonstrated by flow cytometry. AN100226m also interacted with peripheral blood mononuclear cells from dog, pig, guinea pig, ferret, and cynomolgus and Rhesus macaques with similar staining intensity as with human lymphocytes, but not with cells from rat, hamster, gerbil, rabbit, or marmoset. Binding of AN100226m to the α4 subunit of the α4β1 integrin complex was confirmed by flow cytometric evaluation of human T lymphoid and monocytic cell lines expressing various combinations of α4 and β integrin family members, and by immunoprecipitation studies with U937 human monocytic cell lysates. The humanized anti-α4 integrin antibody AN100226 (natalizumab) bound both human Jurkat T cells and guinea pig peripheral blood cells with approximately equal affinity, and could inhibit adhesion of Jurkat cells to mouse L-cells expressing VCAM-1. Saturation concentrations for guinea pig and human T cell natalizumab cell surface binding, and the IC_{50} values for inhibition of T cell adhesion were approximately equal to those obtained for AN100226m, showing the comparability of the humanized version to the parent, murine monoclonal antibody.

Study #: PC032

Volume # and page #: EDR file: STN BLA 125104\000\module4\primarypharm\pc032.pdf
Conducting laboratory and location: Athena Neurosciences, 800 Gateway Boulevard, South San Francisco, CA 94080
Date of study initiation: not specified (final report dated April, 1995)
GLP compliance: no
QAU statement: yes ( ) no (X)
Drug, lot #, and % purity: AN100226m (murine, parent monoclonal antibody) and AN100226 (humanized version; natalizumab), lot number, concentration, formulation, and percent purity not specified in final study report

Methods:

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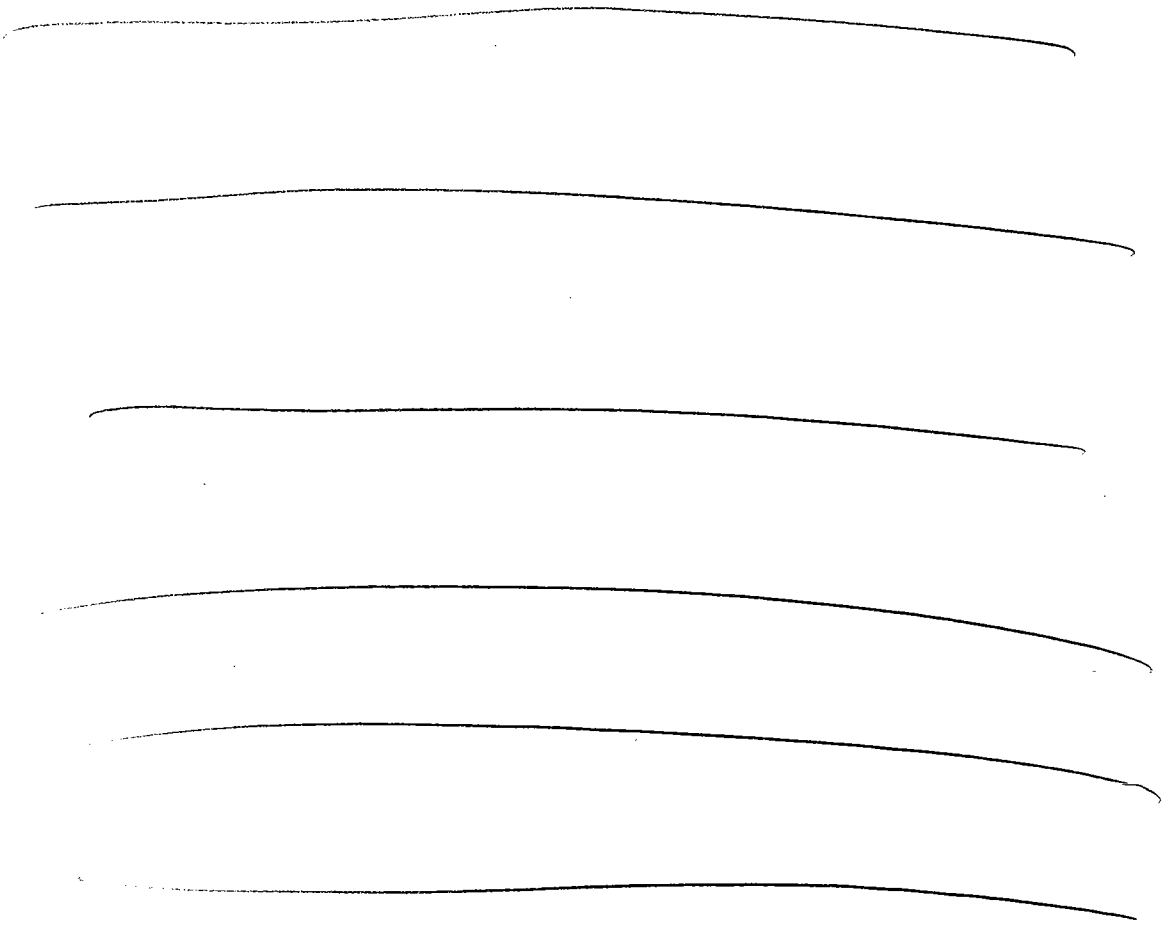

Results: Natalizumab (AN100226m) could inhibit binding of the α4 integrin-expressing human T lymphoid cell lines to activated human or rat vascular endothelial cells, or to murine fibroblasts expressing VCAM-1 by approximately 80 to >97%. Similar results were observed when two additional monoclonal antibodies, HP2/1 and L25, which are known to bind to α4 integrin and inhibit leukocyte adhesion were used. By contrast, pretreatment of Jurkat or Ramos cells with either the non-inhibitory anti-α4 integrin antibody PG49, or the anti-β2 integrin antibody TS18/1, did not significantly decrease adhesion in these assays. Pre-treatment of Jurkat cells with AN100226m, HP2/1, or L25 antibodies also significantly inhibited adhesion to mouse L cells that had been transfected with the cDNA for human α4 integrin, confirming the specificity of AN100226m for this molecule. The results of these experiments are shown in Table 7 and Figure 18, below.

Table 7. AN100226m inhibition of T lymphoid cell binding to activated endothelial cells			
	Percentage of Input Lymphocytes Bound, ± S.D.		
	Rat Brain EC		HUVEC
Antibody Pretreatment	Jurkat	Ramos	Jurkat
None	66 ± 3	40 ± 1	73 ± 8
AN100226m	8 ± 2	1 ± 1	12 ± 3
Inhibitory α4[a]	6 ± 1	2 ± 1	18 ± 1
Non-inhibitory α4[b]	74 ± 1[b]	33 ± 1[b]	n.d.[d]
Non-inhibitory β2[c]	n.d.[d]	n.d.[d]	61 ± 5

[a] HP2/1 (5 μg/ml) used for RBEC experiment; L25 antibody (20 μg/ml) used for HUVEC

[b] P4G9 (negative control antibody against α4 integrin) used for RBEC experiment

[c] TS18/1 (negative control antibody against β2 integrin) used for HUVEC

[d] not done in this assay

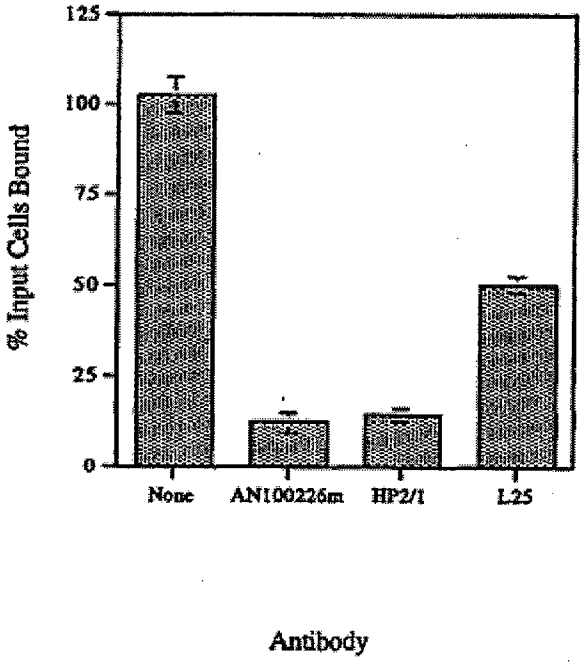

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Figure 18. Inhibition of human Jurkat T cell adhesion to mouse L-cell fibroblasts expressing human VCAM-1. Figure was provided by the sponsor in the final study report.

Pretreatment of human U937 cells with AN100226m completely inhibited their adhesion to inflamed endothelial cells from a rat brain model of EAE. The isotype-matched antibody control OX47 had no effect on U937 adherence. Results are presented as the mean of three replicate

samples, ± S.D. and are shown in Table 8 below, which was provided by the sponsor in the final study report.

Table 8. Inhibition of adhesion of human U937 cells to inflamed blood vessels within sections of EAE rat brain.

U937 Pretreatment	Level of U937 Binding to EAE Vessels (U937 cells bound / reference cell)
None	1.28 ± 0.40
AN100226m	0.01 ± 0.01
OX47 (control antibody)	1.25 ± 0.17

The murine anti-α4 integrin antibody AN100226m bound to peripheral blood monocytes and lymphocytes, but not neutrophils from healthy human volunteer donors, as determined by flow cytometry. Mean fluorescent intensity of AN100226m labeled lymphocytes was comparable to that observed for cells labeled with the anti-α4 integrin antibody HP2/1, as shown in Table 9 below, from the sponsor's final study report. The monoclonal antibody ——— directed against the adhesion molecule Mac-1 (CD11a) reacted strongly with both monocytes and neutrophils in this assay, but much more weakly with two sub-populations of blood lymphocytes. There was no increase in fluorescent intensity over background levels in cells incubated with an irrelevant mouse IgG_1 antibody as an isotype-matched control.

Table 9. Flow cytometry evaluation of AN100226m binding to peripheral blood cells from healthy, human volunteer donors.

	Mean Channel Fluorescence		
	Lymphocytes	Monocytes	Neutrophils
IgG_1 Control	3	7	8
AN100226m	179	163	19
Control Anti-α_4 Integrin (HP2/1)	200	196	21
Anti-Mac-1	3 and 70 (two populations)	704	925

Flow cytometric evaluation of AN100226m binding to peripheral blood mononuclear cells from a variety of different test animal species demonstrated cross-reactivity between the antibody and α4 integrin that was comparable to levels expressed on human lymphocytes. Interestingly, while AN100226m showed a high degree of binding to lymphocytes from both Rhesus and cynomolgus macaques, there was no detectable cross-reactivity with peripheral blood cells from marmosets. Although α4 integrin expression was detectable on the surface of blood mononuclear cells from rabbit, ferret, hamster, and rat using two different anti-α4 integrin monoclonal antibodies, there was no cross-reactivity with AN100226m detected in these species. The results are presented in Table 10 below, which was derived from the data presented in the sponsor's final study report.

Table 10. AN100226m cross-reactivity with α4 integrin expressed on peripheral blood cells from various test animal species

Test Animal Species	Degree of Antibody Cross-Reactivity Relative to Human Cells		
	L25 anti-α4 MAb	HP2/1 anti-α4 Ab	AN100226m MAb
Human	++++[a]	++++	++++
Rhesus monkey	++++	++++	++++
Cynomolgus monkey	++++	++++	++++
Marmoset	(-)	(-)	(-)
Dog	++++	++++	+++ - ++++
Pig	n.d.[b]	n.d.	++++
Rabbit	+++ - ++++	++ - +++	(-)
Guinea Pig	++++	++++	++++
Ferret	n.d.	n.d.	++++
Hamster	++++	++++	(-)
Rat	++++	++++	(-)
Gerbil	(-)	(-)	(-)

[a] The degree of reactivity was scored by comparing the relative intensity of the fluorescent signal obtained, with ++++ indicating a fluorescent intensity in the test species that was 75 to 100% of that for human cells stained in parallel with AN100226m, +++ indicated an intensity 50-74% of the human cells, ++ indicates a 25-49% relative intensity compared to the human sample, + 5-24% of the fluorescent intensity of the human cells, and a score of (-) indicating < 5% reactivity.

[b] n.d. = not determined

The selective binding of AN100226m to α4 integrin was evaluated using various human cell lines that express different combinations of α4 and β integrins, and with the murine L-cell line transfected with human α4β1 integrin complex. The results (mean fluorescent intensity for each sample) of two separate experiments are demonstrated in Tables 11 and 12, which were provided in the sponsor's final study report, below.