Hypophysiary Involvement and Immuno/Growth Depression in Rabies. II. The Participation of Growth Hormone Dysfunction Through the Hypothalamic-Hypophyseal-Thymic Axis in the Bovine Species

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Introduction

Bovine paralytic rabies (BPR) is a dramatic and expensive scourge that affects portions of Mexico, the meso and meridian American subcontinents and the Caribbean. Also the bovine species is the only other one (with the human) for which genetically engineered growth hormone is experimentally available, an essential requisite for our further research as it will become evident in the development of this study.

The combined immunodepression/growth depression effect associated with rabies virus (RV) we have defined as "collapse"[10] and more recently assimilated it to a typical "wasting" syndrome.[14] Expressing weight as the % body weight change from post-inoculation date (PID) 0, disruptions in growth rates were consistently detected. Normal healthy control animals grew linearly with a positive steady % weight change regression coefficient, as an estimate of growth rate. Infected animals with the highest dose stopped growing earlier, later with the lowest dose, and collapse (steeper with the lower doses) was at a steady negative daily rate.[10 12 14] Growth rates, collapse rates and the chronology were predictable and reproducible, and the kinetics already and successfully verified in the rabbit model (Torres-Anjel and Volz, unpublished) as had also been in the mouse,[10] rat,[10 12] and bovine.[12 14]

This investigation was designed to study the feasibility of associating our immuno- and growth-depression data with central growth-hormonal disturbances in the bovine model. Such information was not available from any of the classical bovine rabies studies[1 5 7 8] reviewed in preparation for this study.

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This work is dedicated to the memory of Tadeus ("Tad") J. Wiktor, Dr. med. vet., forceful teacher and permanent source of inspiration.

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Methods

The animals. Seventeen young Holstein bovines weighing 66 ± 15 kg (arithmetic mean ± standard deviation) were purchased from a selected farm or received from a university farm (courtesy of co-author FAM) and allowed to equilibrate in our isolated unit.

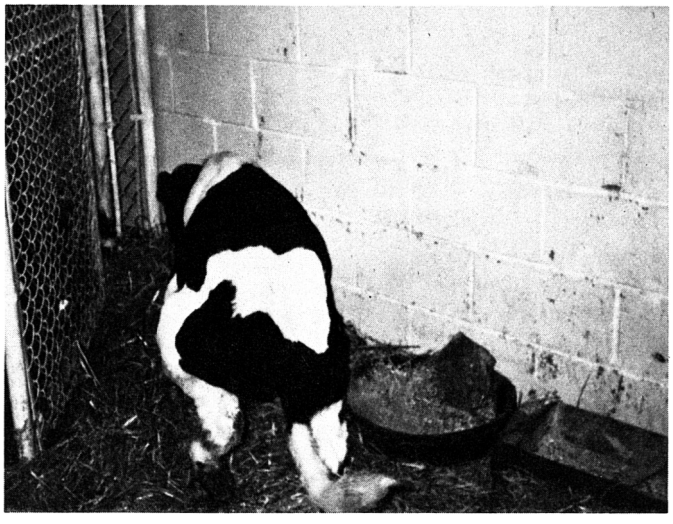
The virus. Isolated in Trinidad (W.I.), it was monoclonal—antibody characterized[13] following the Wistar Institute's panel.[3] Deposited in the American Type Culture Collection under the nomenclature ATCC VR-985 1A, its "biography" has been described elsewhere.[13] It is a BPR rabies virus isolate (BPRV) that also reproduced typical BPR in this study.

Feed deprivation. In this sub-experiment some animals were kept away from solid feed although water was supplied *ad libitum.*

Feed consumption. Although supplied *ad libitum,* solid feed (in this case a weaning-milk replacement feed) was replenished daily in an (weighed) amount known to be higher than the spontaneous consumption, and weighed differentially on the following day to estimate the daily consumption.

Inoculation. The virus was originally injected (RV+) intrathecally (i.t.) at the lumbosacral region (Fig. 1). The no-virus (No RV) controls were parallely injected either i.t. or intra-lingually (i.l.) with an equivalent volume of culture medium. In the original experiment calf C1C8 acted as an uninoculated control. After the completion of the growth/collapse experiment (Exp. 163) the control calf (C1C8) was in turn inoculated as part of the following experiment (Exp. 172). In the following experiments either surviving animals were challenged (Exp. 172) or experimental animals inoculated (Exp. 240) via i.l. (RV+). One animal in each BPRV experiment was left uninoculated as a control (No RV). All animals in Exp. 247 were RV-uninoculated (No RV) and used for other (hormonal, feed

FIGURE 1. Different aspects of experimental bovine paralytic rabies. Upper: Intrathecal inoculation of bovine paralytic rabies. Center: Partial posterior paresia (see Table 2). Lower: Complete paralysis (see Table 2).



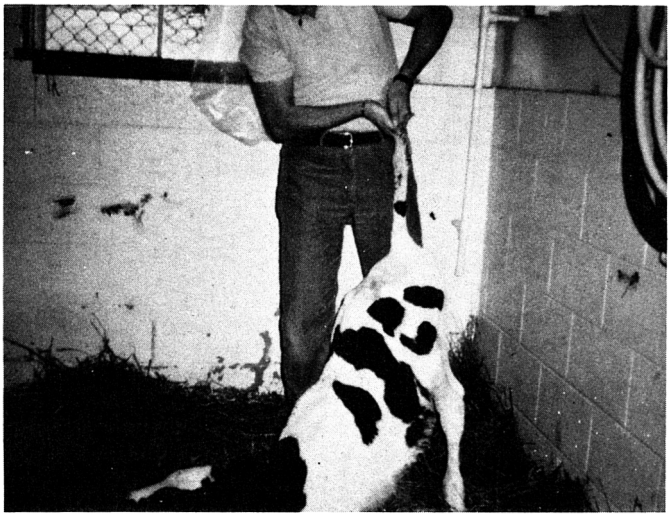


TABLE 1. Chronogram of clinical manifestations in three young bovines injected intrathecally with bovine paralytic rabies virus ATCC VR-985. (Numbers in parentheses indicate reverse day progression, i.e. collapse day = 0).

	Calf 1 (8)	Calf 2	Calf 3
euthanasia-death	(0) pid 15	(0) pid 24	(0) pid 32
thrashing-muscle twitching		(1) pid 23	
brawling		(1) pid 23	
no reaction to stimuli		(1) pid 23	
opisthotonus	(1) pid 14	(1) pid 23	(0) pid 32
lateral recumbency		(2) pid 22	(0) pid 32
loss of nursing ability		(2) pid 22	(1) pid 31
straining	(1) pid 14		(2) pid 30
absent pain response	(1) pid 14	(3) pid 21	(1) pid 31
sternal recumbency	(1) pid 14	(3) pid 21	(2) pid 30
knuckling of front fetlocks	(1) pid 14	(3) pid 21	(1) pid 31
ataxia	(1) pid 14		(1) pid 31
no panniculus	(1) pid 14	(3) pid 21	(2) pid 30
constricted pupils		(3) pid 21	(2) pid 30
hyperesthetic		(4) pid 20	(7) pid 25
increased ataxia		(4) pid 20	(4) pid 28
audible swallowing		(4) pid 20	
ambulates slowly		(4) pid 20	
abdomen "kicking"		(4) pid 20	
grinding		(4) pid 20	
dragging hind claws		(5) pid 19	(4) pid 28
crossing over fetlocks		(5) pid 19	(4) pid 28
difficult swallowing		(6) pid 18	
eye twitching		(6) pid 18	
weakness		(6) pid 18	(4) pid 28
intermittent straining		(6) pid 18	(2) pid 30
ear twitching		(6) pid 18	
skin fasciculation		(7) pid 17	(6) pid 26
knuckling of rear fetlocks		(7) pid 17	(8) pid 24
hypermetria		(7) pid 17	
slight ataxia		(7) pid 17	
reluctancy to stand		(17) pid 7	(4) pid 28
ears held back	(1) pid 14	(17) pid 7	(2) pid 30
increased blinking		(17) pid 7	
shivering		(17) pid 7	
scratching		(17) pid 7	(8) pid 24
standing alone		(16) pid 8	
inoc. with rabies virus	(15) pid 0	(24) pid 0	(32) pid 0

deprivation) manipulations.

Titration of the wasting phenomenon. Representative samples of the RV were diluted in our cell culture medium and titrated for the growth disruption effect in the weanling rat model by intracranial (i.c.) injection.[13] The same approach was taken by studying the experimental calves as a pool and with respect to the BPRV dosage/kg, different in each case by definition.

Virus dosage. The dosages applied were consistently $10^{1.5}$ to $10^{2.2}$ ED_{50}/kg rat/kg calf for either the i.t. or i.l. routes.

Growth hormone. The terms somatotropic hormone (STH) and growth hormone (GH) will be used interchangeably in this work. A prefix will indicate the species the hormone originated from bovine (b), rat (r), etc. The bGH was radioimmunoassayed (RIA) in sequential serum samples[14] by a technique described elsewhere.[6] The immuno-

TABLE 2. Affection of growth (estimated as body weight changes, %) by rabies virus effect upin linearly growing calves. Calves C1C8 and CC were uninfected (blank inoculated) controls. Calf M1 was feed-deprived, M2 normal rabbit serum treated and M3 anti-rat growth hormone (rabbit) serum treated. The remaining ones (+) were rabies virus infected/affected. Numbered animals were inoculated intrathecally and lettered animals intralingually.

Calf Code	Initial weight (kg)	Coefficients	
		Regression*	Correlation**
	Positive growth		
C2+	53	+1.5929	+0.9739
C3+	59	+1.5954	+0.9934
CA+	69	+1.5123	+0.9870
CB+	70	+1.2647	+0.9870
M2	90	+0.9448	+0.8600
M3	63	+0.8069	+0.7800
C1C8	41	+2.2503	+0.9885
CC	79	+1.4120	+0.9970
Mean	65.5	+1.4224	+0.9485
(SD)	(15.2)	(0.4447)	(0.0808)
n = 8			
	Negative growth (rabies collapse)		
C2+	53	−3.2788	−0.9293
C3+	59	−2.6229	−0.9293
CA+	69	−1.9643	−0.9600
CB+	70	−4.4000	−0.9680
Mean	63	−3.0665	−0.9467
(SD)	(8)	(1.038)	(0.020)
n = 4			
M1	113	−2.3704	−0.9400

*Based on BODYWEIGHT changes % (CHANGE)
**Equal (to the third decimal) for either body weights (WEIGHT) or CHANGE

staining *in situ* technique permitted evaluation of bGH production in the STH producing acidophil cells in the adenohypophysis (alpha-pituicytes). See below under *Immunostaining.*

Monitoring of infection/affection. Several parameters were followed up in detail in different groups of experimental animals. Three animals were carefully followed up for clinical manifestations (Tables 1 and 2) and clinical pathology observations (Table 3). Three other animals were monitored for daily feed consumption by a differential feed weight procedure. The feed weight (FEED) was computed as a coefficient with metabollic body size (MBS, body WEIGHT in kg elevated to the 0.75 power) as a denominator to eliminate the possible effect of "less feed consumption because of lesser body size." All animals were body WEIGHT monitored daily or bi-daily from equilibration to necropsy time and PERCENT body weight changes were calculated based on the WEIGHT at post-inoculation 0 which was considered as 100%. The oscillation in weight was calculated as PERCENT -100 or percent body WEIGHT reduction (PERRED). The spleen was measured (length x width = SPLDIM or approx. spleen area in cm^2) and weighed (SPLWT). The SPLWT/WEIGHT at necropsy (NCWT) % ratio (PERATIO) and the SPLDIM were calculated as indicators of immunostatus. All regressions were calculated with PERCENT or PERRED as Y upon the time in DAYS (X), either pre- or post-inoculation (PID).

Immunostaining. The diagnosis of RV in the affected animals has been the subject of other publications.[11 16] The application of not only anti-RV but anti-GH *in situ* staining and the staining for (non-immune) endo-peroxidase were applied in this study and are described briefly as follows:

The immunostaining techniques were based on the direct immunofluorescence (IF) and on the III steps immunoperoxidase (IP) peroxidase-anti-peroxidase (PAP) that we will call IPP. The basic technology as applied to RV anti-nucleocapsid has been described in our former publications.[10 11 13 16] The anti-GH IPP followed an identical protocol from the secondary antibody on. The primary antibody in the case of the GH/STH was a hyperimmune anti-rSTH rabbit (Ra-rGH) polyclonal antiserum produced throughout one year's hyperimmunization of New Zealand white rabbits with adjuvanted (Freund's complete) rSTH (National Pituitary and Hormone Program, Baltimore, MD USA). The rabbit primary antibody, either against RV or GH, was conjugated with either fluorescein (FITC) or rhodamine B (RBITC) for direct IF.

Because our anti-RV IPP is based on development with a diamino-benzidine (DAB) substrate, one of our consultant pathologists (co-author LK) expressed the presence of RV antigen in terms of DAB. His nomenclature has been respected. Thus in the immuno-histopathological description of lesions DAB is equivalent to BPRV antigen.

Inoculation with anti-GH serum. Animals in this experiment received, intravenously (i.v.), anti-rGH prepared in the rabbit (as shown above) and titrated in the rat. Controls (No RV) for this experiment received equivalent volume of normal rabbit serum (NRS) by the same via (Exp. 247).

Endogenous autochthonous peroxidase staining. This technique has to be carefully differentiated from immunoperoxidase (IP) staining. It consisted of the utilization of the same substrate as utilized in the IP reaction but without any of the previous steps. It is a cytochemical, and not an immunochemical, reaction that will detect the presence of the (endo-) peroxidase enzyme as characteristically present in erythrocytes, polymorphonuclears, macrophages, and other specific reticuloendothelial cells. A detailed protocol for the management of the DAB-substrate has been published elsewhere.[16]

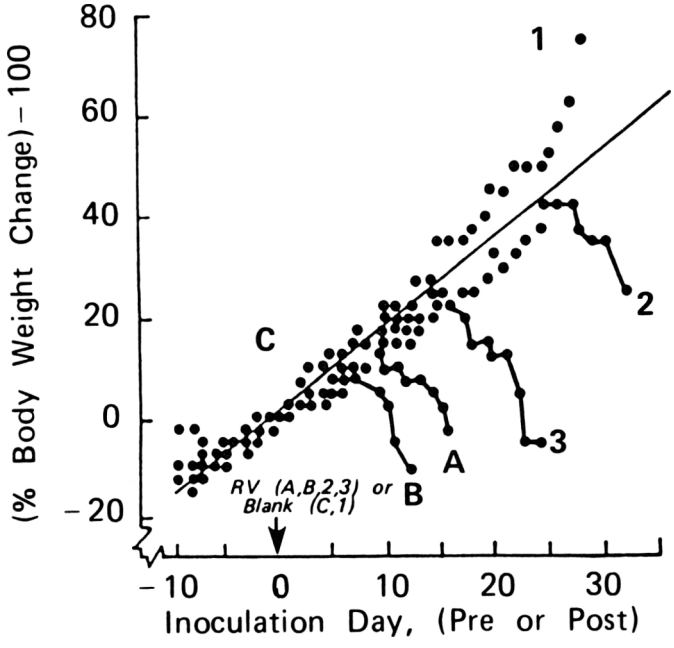
Data Handling. All data were stored through a terminal connected (TSO) to the UMC's mainframe computer. Data were processed by a BMDP[3] software statistical package as described before,[12] and by Macintosh™ Plus hardware (Apple

TABLE 3. Summary of outstanding clinical pathology parameters in young bovines after rabies virus inoculation.

	Post-Inoculation Date										
	-3			0			15			19	47(*)
	1	2	3	1	2	3	1	2	3	1	1
PCV %	18	29	28	17	28	28	20	28	25	26	33
HgBg/dL	6.2	10.7	10.7	6	10.1	10.6	7.4	10.7	10.1	9.5	12.9
WBC**/mm^3x10^3	5.7	9.7	8.4	6.7	11.5	6.0	6.6	13.2	10.9	8.0	14.3
Seg. Neut. %	32	42	66	33	42	21	65	51	25	43	70
Seg. Neut. abs.**	1.82	4.07	5.54	2.21	4.83	1.26	4.29	6.73	2.73	3.44	10.0
Lymphocyte %	57	47	28	60	52	78	34	47	70	53	24
Lymphoc. abs.**	3.25	4.56	2.35	4.02	5.98	4.68	2.24	6.20	7.63	4.24	3.43
Monocyte %	11	9	4	6	1	1	1	2	4	4	4
Monocyte abs.**	0.63	0.87	0.34	0.40	0.12	0.06	0.66	0.26	0.44	0.32	0.57

* The "control" No. 1 calf (no virus), after being so for calves No. 2 and 3 (Exp. No. 163) (inoculated/infected) and after their termination was also inoculated/infected as part of a further experiment (No. 172) and died on d 47.
** Per $mm^3 \times 10^3$

FIGURE 2. Pooled growth and collapse statistics for 2 uninoculated (No RV) and 4 bovine paralytic rabies virus inoculated (RV+) calves. The regression statistics of these graphs are summarized in Table 1.
Note that numbered animals were inoculated intrathecally (see Figure 1) and lettered animals intralingually, with the latter giving the shorter incubation times.



Computer Co., Cupertino, CA 95014) with StatWorks™ and Graph™ software (Crickett, Philadelphia, PA 19104).[9A]

Results

The clinical picture. The detailed monitoring of symptoms in three of the animals studied at great length is shown in Table 1 and Fig. 1.

Body temperature. Results were quite uniform with a tendency for hypothermia to occur at RV-collapse time. Actual data are not presented.

The collapse. A summary of the statistical growth parameters as studied in 6 animals (2 controls) is seen in Fig. 2 and Table 2.

The clinico-pathological parameters. A summary of the observations is shown in Table 3.

Titration of the wasting phenomenon. The ability of the BPRV to produce the wasting phenomenon was described by means of two statistical approaches. One was to plot the ratio of presentation of wasting (number of affected animals/total animals inoculated in a particular experiment) in the abscissa and the log of the dose of BPRV (expressed as volumen of BPRV/body weight of the animal in kg at inoculation time) in the ordinate. The result was a significantly positive linear regression with a coefficient of 1.6, correlation of 74% and statiscial significance of $p < 0.001$. This first approach was applied to the titration of the BPRV in rats (Fig. 2) prior to the bovine inoculations; and to the actual titration of the BPRV in the calves. The regression parameters for the rat regression were 2.6, 60% and < 0.001 (Fig. 3).

FIGURE 3. The linear regression analysis of the different titrations applied to the wasting syndrome phenomenon effect.
Upper: Bovine paralytic rabies virus effect in the weanling rat linear regression model. Ordinate's dose is ml virus/kg body weight rat.
$Y = 2.607\times - 4.809$; $r = 0.602$; $p < 0.001$
ED50 calculated from graph = 10-3.64 ml per kg rat
Center: Bovine paralytic rabies virus effect in the linearly growing calf regression model. Ordinate's dose is ED50/kg rat as applied in the calves.
$Y = 1.606\times + 0.563$; $r = 0.739$; $p < 0.01$.
ED50 calculated from graph = 101.36 ED 50/kg rat/kg calf
Lower: Rabbit anti-rat growth hormone as titrated in the linearly growing weanling rat.
$Y = 2.389\times + 0.208$; $r = 0.416$; $p < 0.05$
ED50 calculated from graph = 101.61 ml per kg rat

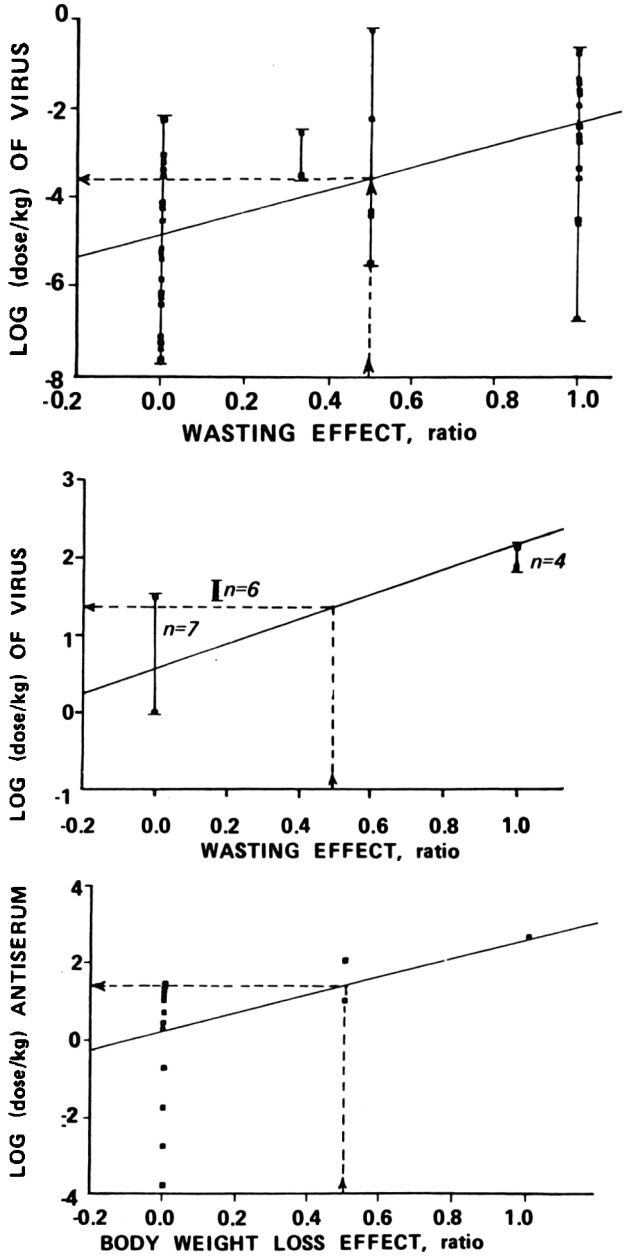


FIGURE 4. The relationship between bovine paralytic rabies virus (ATCC VR-985) dosage and day (post-inoculation) at which maximum growth (plateau) was obtained in calves.
$Y = -0.102\times - 2.384$; $r = 0.332$; $p < 0.002$

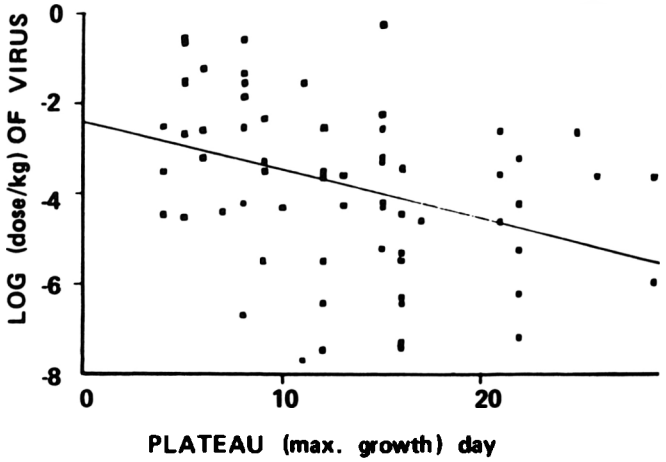
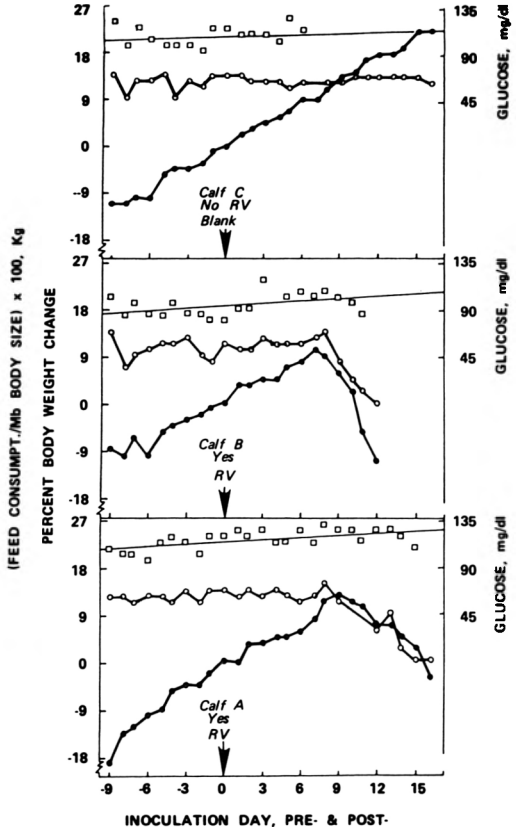


FIGURE 5. The relationship between growth expressed as percent body (daily) weight increase, the consumption of feed (as related to metabolic body size) and plasma glucose levels in experimental bovine paralytic rabies. The linear regression statistics of growth and collapse are summarized in Table 1.
Note that neither feed or glucose levels are affected prior to, but only together with, "thriftlessness" and growth collapse.
Legends. Growth (dark circles); feed (light circles); glucose (squares).



In titrating the effect of the Ra-rGH (see above) in the rat (Fig. 3) the body weight loss phenomenon was the measured criterion. The regression parameters were +2.4, 42% and < 0.48.

The other (Fig. 4) was to plot the day at which maximum body weight (PLATEAU) was attained, in the abscissa, and the log of the dose of BPRV in the ordinate. The result in the rat was a significantly negative linear regression with a coefficient of -0.132, correlation of -0.332 and $p < 0.002$.

The RV× collapse vis-a-vis feed consumption and plasma glucose. The results of these detailed studies, carried out individually in each of 3 animals, are presented in Fig. 5. As it may be seen the plasma glucose data did not follow any particular pattern with respect to either the linear positive or negative growth portions of the curve. The FEED/MBS coefficient remained stable up until the presentation of the decrease in PERRED. From then on, and not before, there was a reduction in FEED/MBS consumed that followed parallely, but that did not *precede,* the stoppage of growth.

Immunostaining and Histopathology

The immuno-organs. The results of the study of the immunotopography of the thymus and the spleen may be seen in Tables 4 and 5. The most remarkable set of thymus data, corresponding to Exp. 240, are presented in Fig. 6. The most remarkable features of spleen are illustrated in Fig. 7.

The hypothalamus. Immunostaining for RV showed marked RV+ immunoreactivity in the hypothalamus. Several aspects of neuronal infection in the hypothalamus are illustrated in Fig. 8.

The hypophysis (pituitary gland). Immunofluorescence and immunoperoxidase of the adeno-hypophysis presented cells infected *with remarkably* high positive levels of RV (Fig. 9).

Growth hormone. The presence of GH in GH-producing alpha-pituicytes of the adenohypophysis was evaluated by an end-point titration of the anti-rGH primary antibody in an *in situ* immunostaining (IF and IPP) system. There were significant differences (markedly lower in the RV+ animals) in the titration, in the intensity of the staining and in the acutal number of GH-immunoreactive cells both in the "crowded" as well as in the "scattered" GH-producing areas of the adenohypophysis (Table 7). The marked differences in anti-GH immunostaining of GH-producing alpha-cells of the adeno-hypophysis between No RV controls and the RV+ infected-affected animals is shown in Fig. 10.

Trigeminus-(V Cranial pair)-Gasser ganglia. Using IF (fluorescent anti-nucleocapsid antibody) to detect the presence of rabies antigen, sections of the trigeminum (Gasser ganglia) demonstrated viral infection of the area. Infection of this site indicates both the ascent (centripetal) of the virus from inoculation site and the descent (centrifugal) from the CNS. It has become our preferred site for immuno-

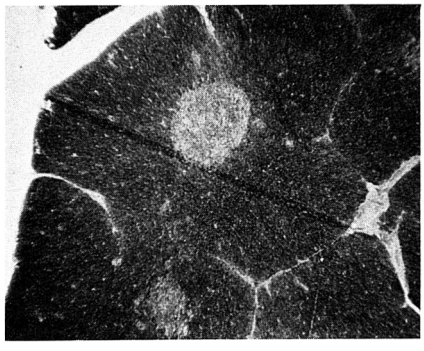
TABLE 4. Summary of immunostatus based on histo-immunotopography of spleen and thymus in rabies virus (RV) infected-affected (RV+) vs No RV calves corresponding to photographs presented in Figures 6 and 7.

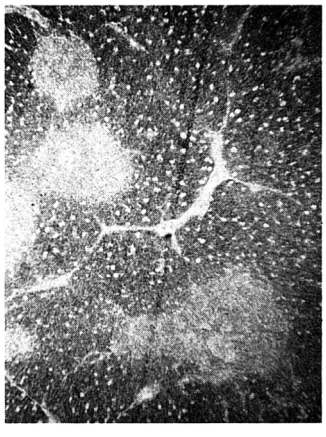
ORGAN	RV+	No RV
SPLEEN		
Follicles	Small, inconspicuous Few or no germinal centers	Well developed Most with germinal centers
Periarteriolar sheaths (PALS)	Small, hypocellular Few or no mitosis	Densely cellular mixture of small and large lymphocytes with 3 to 5 mitosis per 400x field
Neutrophils	Numerous in perifollicular red pulp	
THYMUS		
Lobules	Shrunken	Expected size for a healthy animal
Cortex vs medulla	Poorly differentiated because of cortex hypocellularity and relative increase in intersticial tissue	Well differentiated Cortex occupies a greater proportion of the thymus. Densely cellular. Composed predominantly of small lymphocytes with few scattered reticular cells
Hassel's corpuscles	Adequate numbers	
Corpuscles Eosinophils	Lightly infiltrate the medulla and interlobular septa	

TABLE 5. Summary of immunostatus based on histo-immunotopography of spleen and thymus in calves intervened in several manners as controls (No RV) to rabies virus experiments. NRS = normal rabbit serum; Ra-rGH = rabbit-origin anti-rat growth hormone; DEP = feed deprived.

ORGAN	NRS	Ra-rGH	DEP
SPLEEN	7186E Normal periarteriolar lymphoid sheaths (PALS) and follicles. Moderate number of neutrophils in red pulp.	7186H The only apparent difference was a lesser ratio of cortex to medulla than in 7186D. Ratio was greater than in 7186A.	7186B Some periarteriolar lymphoid sheaths (PALS) and follicles resembled those in 7186E. Other PALS were less densely cellular with small follicles. Light scattering of nuclear debris in some follicles.
THYMUS	7186D Normal thymus with densely cellular cortex, distinct corticomedullary demarcation and light eosinophil infiltration of medulla and interlobular septa.	7186G Similar to 7186B.	7186A The only detectable difference from 7186D was a relative decrease in ratio of cortex to medulla. This was most easily observed by gross examination of the slide.

FIGURE 6. The different stages of thymic atrophy/involution/ depletion during experimental bovine paralytic rabies virus infection/affection in weanling calves. (119X; 1 cm = 84 um)
Upper: Healthy uninoculated (No RV) control calf.
Lower: Infected/affected calves with bovine paralytic rabies virus]
Lower left: Early stages of thriftlessness, wasting and growth "collapse".
Lower right: Late stages of the deep growth collapse.





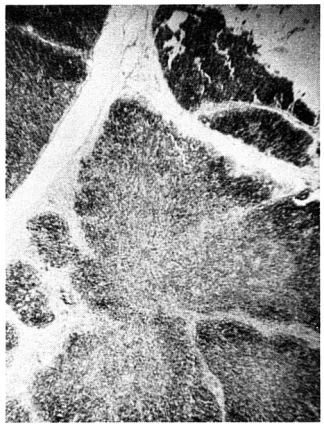
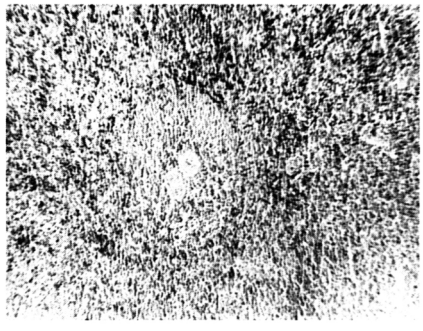


FIGURE 7. Features of the spleen in uninfected (upper) healthy controls (No RV) vs a paralytic bovine rabies virus (lower) upon staining with diamino-benzidine substrate for the enzyme peroxidase. (340X; 1 cm = 2.94 um)
Upper: Note the very healthy appearance of the periarteriolar lymphoid sheath (PALS) which is characteristically rich with T-lymphocytes which are by definition negative to the enzyme perovidase. Also the absence of obvious fibrous connective splenic tissue.
Lower: The PALS is occupied mostly by peroxidase+ obviously non-T-type cells and fibrous tissue preempting splenic lymphoid tissue. Both features indicate a (T-cell) lymphoid depletion.



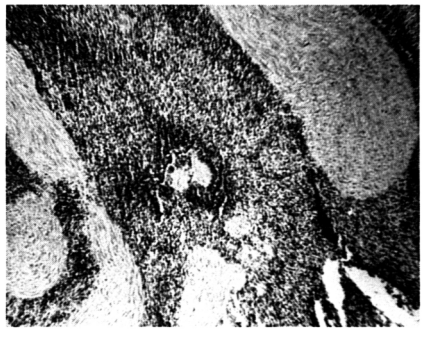


FIGURE 8. Anti-rabies virus nucleocapsid's immunoreactivity (peroxidase-anti-peroxidase, PAP) in the hypothalamus of calves experimentally infected with bovine paralytic rabies. (530X; 1 cm = 6.5 um)
Arrows indicate the presence of rabies virus.
A more detailed study of the invilvement of the hypothalamus in rabies was published under ref. no. 15.

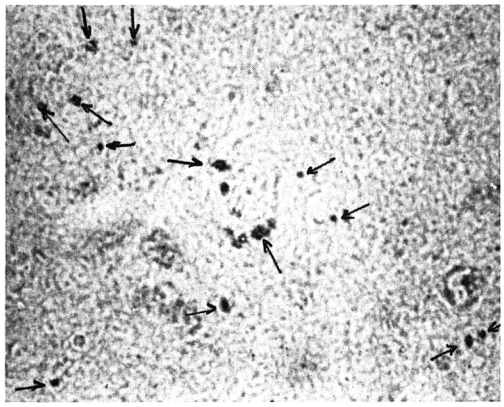
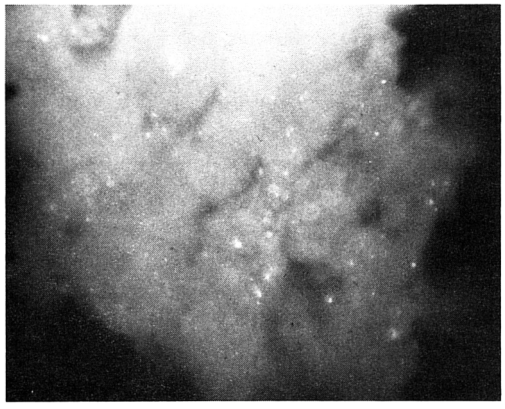


FIGURE 9. Anti-rabies virus nucleocapsid immunostaining in the adenohypophysis of calves inoculated experimentally with bovine paralytic rabies virus. (3400X; 1 cm = 2.94 um)
Upper: Fluorescent (FITC)
Lower: Peroxidase-anti-peroxidase (PAP)
Notice the multiple cytoplasmic minute foci appearance of the adenopituicytes (secretory epithelium) activity infected with RV.



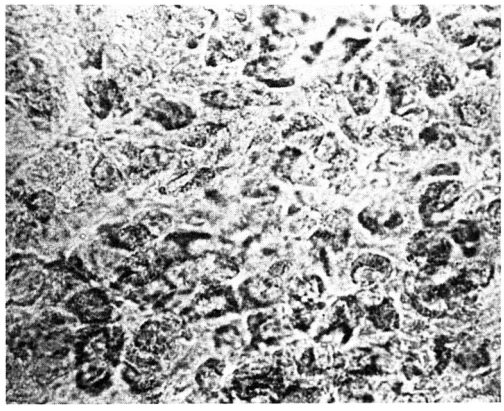


TABLE 6. Summary of experimental and immunostatus (spleet) data for bovine.

Exp. No.	Anim No.	RV Inoc. I* Y+	RV Inoc. I* N++	RV Collap. Y	RV Collap. N	RV Inoc. II** Y	RV Inoc. II** N	Spleen SPLDIM	Spleen PERATIO
163	C1(D)		X	X			X	ND	ND
	2(AL)** (Dead)	X		X			X	221	0.428
	3(AL)	X		X			X	225	0.210
172	C1(AL)		X		X		X	240	0.343
	C2(AL)	X			X		X	293	0.352
	C3(D)	X			X		X	213	0.148
	C4(AL)	X			X	X		244	0.247
	C5(AL)		X		X		X	208	0.191
	C6(AL)	X			X		X	252	0.288
	C7(D)	X			X	X		181	0.159
	C1C8(D)	X						ND	ND
240	A	X		X			X	218	0.270
	B	X		X			X	198	0.240
	C		X		X		X	305	0.340
247	A(D)		X		X		X	268	0.288
	B(NRS)		X		X		X	234	0.204
	C(AGH)		X		X		X	204	0.246

Experiment 163 was inoculated intrathecally (i.t.)
Experiment 172 was inoculated i.t. and challenged intralingually (i.l.) and included Calf C1 from Exp. 163 (C1C8). See text in Materials and Methods.

Experiment 240 was inoculated i.l. Experiment 247 was No RV-inoculated.

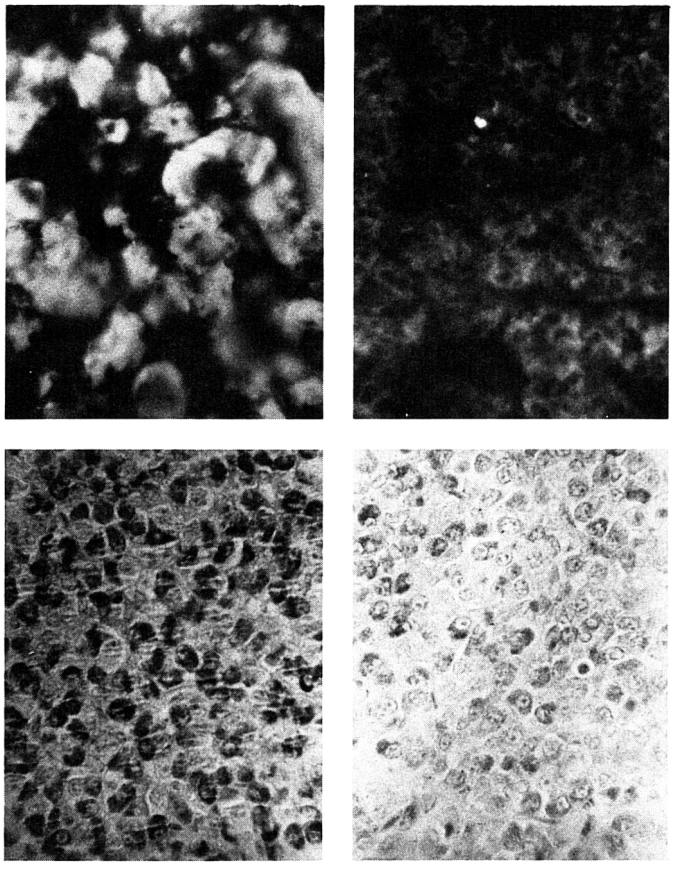
+ Y = yes; ++ N = no
AL = food and water ad libitum; D = food and water deprived; NRS = normal rabbit serum; AGH = rabbit anti-rat growth hormone.

TABLE 7. Titration of in situ cell bound anti-somatotropic hormone by immunostaining end point determination as an estimate of somatotropic hormone in the bovine adeno-hypophysis: comparison of rabies virus infected/affected (wasted) (RV+) animals vs non-infected blank inoculated (RV−) controls. See Figure 10.

Calf No.	Adeno Titer (log_{10})	Signif. Difference+	GH-producing cells in the scattered portion of the hypophysis % STH Prod. Cells
246 A RV+	-2.8		1-5
		N.S.	
9246 B RV+	-2.8		1-5
		***	
260 C(o) No RV	-3.4		25

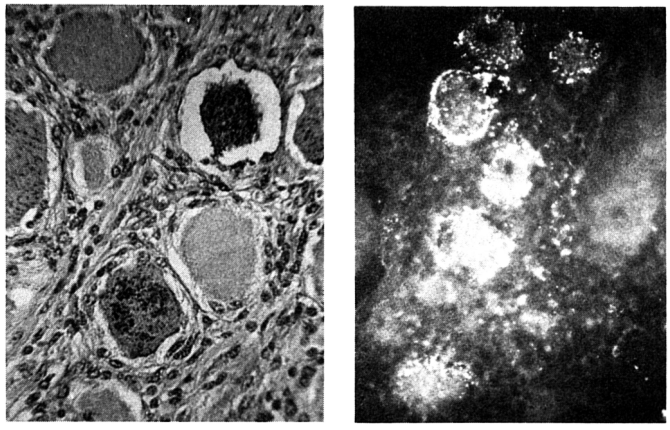
+ $p < 10^{-*}$

FIGURE 10. Comparative anti-growth hormone (GH) or somatotropin (STH) immunoreactivity in the adeno-alpha-pituicytes of healthy uninfected (No RV) (right) vs bovine paralytic rabies infected (RV+) wasting (left) calves. (3400X, 1 cm = 2.94 nm)
Upper: Flourescent (FITC).
Lower: Peroxidase-anti-peroxidase (PAP).
Note the very remarkable diminution in the immunoreactivity of the hypophysis in the RV+ animals, not only in terms of intensity of the reaction (predicting the amount of GH/STH being produced) but also in terms of the relative number of reacting adeno-alpha-pituicytes (predicting which adeno-alpha-pituicytes remain that actually capable of producing GH/STH).
The photographs illustrate the prior-to-end point titration of GH/STH in adeno-alpha-pituicytes as calculated in Table 7.



histo-cytological observations as it is a nervous cranial trunk (V pair) with both motor and sensory capabilities, and provides tongue, eye, and mandible (to and/or from) innervation.[10 11] It also provided at one sight the best overall picture of the different stages of ganglion cell infection, affection, invasion and destruction. Sections of trigeminus stained by IP showed a few ganglion cells being phagocytized or undergoing necrosis. Nuclei were absent and the cytoplasm was undergoing lysis. Small round cells have considerably increased in numbers around the infected ganglion cells. These cells were probably lymphocytes.

FIGURE 11. Anti-nucleocapsid immunoreactivity of rabies virus in the trigeminus nerve's (TN or V cranial pair) Gasser ganglia (GG) of calves experimentally inoculated with a bovine paralytic isolate (1530X; 1 cm = 6.5 um)
Left: Fluorescent (FITC)
Right: Peroxidase-anti-peroxidase (PAP)
The TN's GG were, together with the cerebellum the preferred diagnostic site in this bovine study. A detailed study of the TN GG's in rabies diagnosis was published as Ref. No. 11.



In the trigeminal (Gasser) ganglia stained by IPP two-thirds of the ganglion cells contained DAB granules in the cytoplasm. The degree of staining ranged from a slight brown discoloration of the cytoplasm due to dust-like (minute) particles to a dense brown color with coarse heavily stained granules. The DAB staining tended to be diffuse and equally distributed throughout the cytoplasm of the stained cells. In the trigeminal (Gasser ganglia), many of the large motor neurons were undergoing necrosis. Nuclei were absent and the cytoplasm was undergoing lysis. Spaces previously occupied by neurons were being filled with lymphocytes. There was marked swelling of Schwann cells in the nerve bundles. Some of the bundles were being infiltrated with lymphocytes. Fig. 11 illustrates the findings associated to the trigeminal gasserian ganglia in this study. A more detailed study of this diagnostic site in rabies studies has been presented elsewhere.[11]

Brain hippocampus. The Ammon's horn stained by IPP showed that more than ½ of the neurons in the hippocampal gyri were stained with DAB granules. The long dendritic processes were conspicuous because they contained DAB granules. The dendritic cell layer in the deep polymorphic cells were free of RV antigen. The hippocampus neurons in the hippocampal gyrus were undergoing degeneration changes. The area was infiltrated with lymphocytes or glial cells. There appears to be a light, diffuse gliosis. A number of the vessels were conspicuous because of swollen endothelial cells and a minimal amount of perivascular cuffing. Neurons in scattered areas showed both satellitosis and neuronophagia. The latter neurons were necrotic. There might have been a slight gliosis. No Negri-bodies were ever encountered by regular histo-staining alone in this "favorite" site or for that

FIGURE 12. Four different magnifications of the anti-RV three steps immunostaining peroxidase-anti-peroxidase) of the hippocampus (Ammon's horn) in experimental bovine paralytic rabies.
Upper left: 340X (1 cm = 29.4 u)
Lower left: 1530X (1 cm = 6.5 u)
Upper right: 850X (1 cm = 11.2 u)
Lower right: 3400X (1 cm = 2.94 u)

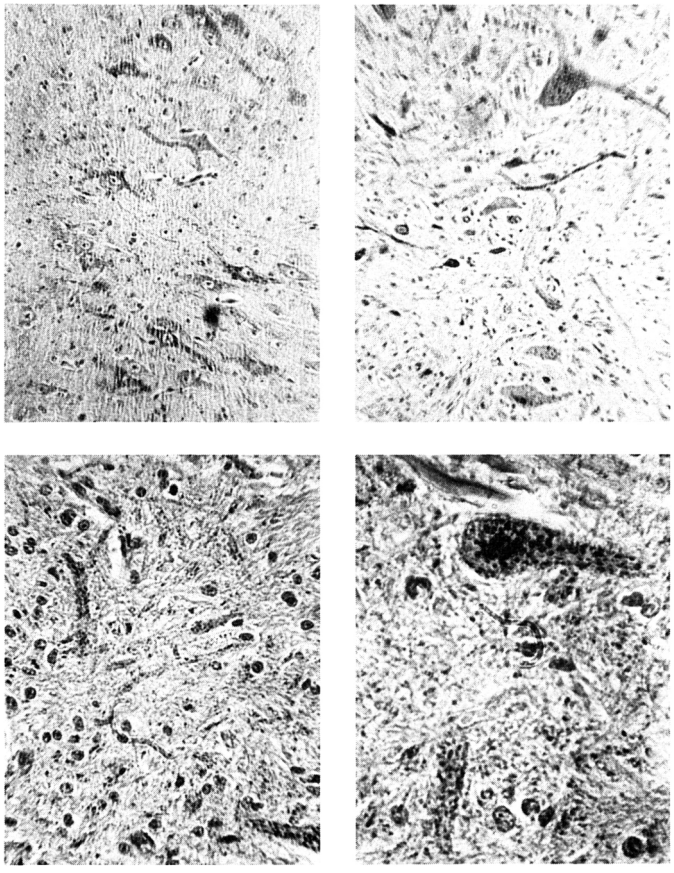
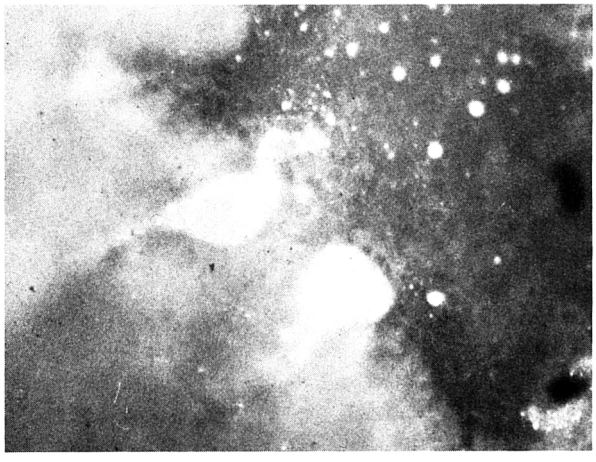


FIGURE 13. Bovine paralytic rabies virus immunoreactivity as observed in the Purkinje cells of the cerebellum (3400X; 1 cm = 2.94 um) during experimental infection. Together with the trigeminus nerve's Gasser ganglia, the cerebellum constituted the best diagnostic sites.



matter any other sites during this study.

An example of anti-RV IPP immunoreactivity in the hippocampus (Ammon's horn) is shown in Fig. 12. The hippocampus was a relatively unimportant diagnostic site in this study in bovines.

Cerebellum. By IPP staining in the cerebellum, the peripheral molecular layer and the granular layer were diffusely and heavily stained with the DAB pigment. Other portions of the same slide show no staining of the cells in the granular layer. Two-thirds or more of the Purkinje cells were stained with DAB. The staining ranged from a light dusting of the cells to heavy granules which gave the cells a heavy diffuse brown color. There was some DAB staining of the outer molecular layer. The staining tended to be in long linear columns suggestive of fiber tracts. Except for an occasional large neuron there was practically no staining in the molecular layer of the cerebellum. The deeper fiber tracts of the cerebellum contained an occasional stained cell. Only one cell per 4 or 5 high power fields contained rabies antigen pigment. An example of the anti-RV IF FA of the cerebellum, the preferred RV diagnostic/confirmatory site in this study in bovines, is shown in Fig. 13.

Adrenals. In the adrenal, *per se* no RV was seen. In ganglia adjacent to the gland some infiltration by lymphocytes and within them a few neurons contained a small amount of RV antigen described as DAB. No other remarkable findings were ever registered.

The hypothalamic-hypophyseal-thymic axis (HHTA). The evidences obtained through this and other of our studies which suggest central hormonal dysfunction are listed in Fig. 14. A schematic representation of how these and our other results may be interconnected through GH in the HHTA is enunciated also in Fig. 14.

Discussion

A detailed follow-up and illustration of the clinical signs had as a purpose, and accomplished, to demonstrate that experimental BPR was clinically undistinguishable from the field disease. BPR was reproduced with a well-established tissue cultured BPRV strain which, although of bovine origin, had gone as a matter of definition through *in vivo* mice and rat passages and *in vitro* baby hamster kidney (BHK) (Torres-Anjel, unpublished).

The histopathology of the experimental BPRV was also typical and homologous with what is expected in the natural disease. Innovatively, the trigeminus was found to be an ideal diagnostic site particularly important when the hypophysis is to be studied since the the trigeminus is charac-teristically juxtaposed to this gland and may be conveniently utilized as a marker. The marked presence of BPRV in the cerebellum coincided with obvious motor and equilibrium changes in the patients. The hippocampus, although utilizable in determination of BPRV diagnosis, was not the most important site when compared to the TN and the cerebellum.

The kinetics of the BPR's wasting syndrome and collapse

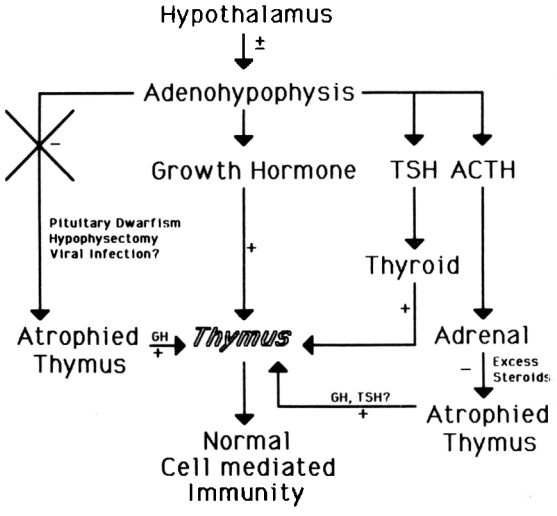
FIGURE 14. Objective (upper), suggestion (center) and postulation (lower) of the role of growth (and other?) hormone dysfunctions as affected by rabies virus, through the hypothalamic/hypophysiary/thymic axis.
It is postulated that RV acts upon this axis by its tendency to infect the hypothalamus and the hypophysis (adeno-) thus impairing by one, or the other, or both mechanisms the production of somatotropic- (growth-) hormone.

Objective:

Determine if growth hormone dysfunction plays a role in the pathogenesis of rabies.

Evidence Suggesting Central Hormonal (GH) Dysfunction:

1) Hypothalamic infection
2) Hypophysary infection
3) Dramatic growth reduction
4) Immune depression



were identical in general appearance, although of course different in magnitude, to those which had been observed in the other RV animal models: murine, rat and rabbit species. Of general interest was the fact that the i.l. route turned out to be more efficient in terms of ED_{50}/kg and incubation times, than the i.t.s's. The injection into the tongue being a mass of muscle tissue would seem to better simulate a parenteral infection. However, because of its extremely abundant motor innervation, the tongue turned out to be neurologically "closer" to the target areas of the brain with the result that the RV infection was easier when put into the tongue than when the RV was placed directly into the CNS, presumably the cerebrospinal fluid (CSF), during the i.t. injection.

The clinical-pathological data seemed to indicate at least some abnormality in the lymphocyte population in some of the RV+ animals which of course was better demonstrated by the very marked thymic topographic abnormalities and then by the obvious lesions in the spleen which further demonstrated not only lymphoid, in general, but T-cell in particular, depletion.

The infection of the hypothalamus was particularly obvious when specimens were studied with the IPP technique since this is a staining that is 10 to 100X less sensitive to the IF routinely used in RV diagnosis. Under the IPP conditions, as compared to IF where at least some degree of RV presence is observed throughout the brain, those areas that were positive corresponded to areas where the concentration of RV was particularly remarkable. Such an area was the hypothalamus. The hypothalamus controls growth through the hypophysis and via hypothalamic "hormones": GH-releasing factor (GHRF) and its feedback shunt through GH-inhibiting factor (GHIF) or somatostatin. The hypothalamus is also responsible for body temperature control and maintenance. That the hypothalamic infection may be responsible for changes in the neurotransmitters associated with hypophysis function and/or GH/STH secretion needs to be clarified particularly since the adenohypophysis itself was found to be remarkably, and surprisingly, in a state of active infection (see below).

The specific infection of the adeno-pituicytes with RV, BPRV in this case, continued being one of the most puzzling and innovative developments of our work, particularly since parallel studies *in vitro* have shown that the RV-infection has also cytological and functional consequences for the cells and their ability to produce GH/STH (and other hormones).[17] The infection issue became particularly significant since it coincided with a remarkable diminution in the production of GH/STH by the adeno-alpha-pituicytes in the RV+ animals as well as in the relative number of GH/STH-producing adeno-alpha-pituicytes in the hypophysis of the BPRV+ infected/affected animals. Our studies will proceed with the study of actual levels of GH/STH in the plasma which is the most orthodox procedure. However, since the level of GH/STH was in the level of ng/ml, that is, approx. in the area of 10^{-9} or parts per billion (ppb), any changes in the plasma volume or hydration status of the animal would have a very significant influence in the level of the determined GH. (Such changes are obviously very much within the realm of the wasting syndrome being studied here.)

When control (No RV) animals were feed-deprived, some changes in immuno-histo-topography were accomplished, although they were comparatively very minor with respect to the full RV+ effect. That is, feed deprivation alone could not justify the marked immunodepletion (immuno-histo-topography changes) observed in the RV+ animals. On the

other hand, some important thymic changes, similar to what was observed in the RV+ animals, were obtained when calves were injected intravenously with Ra-rGH. Ancillarily, the effect of heterologous (r)GH in the bovine was demonstrated.

Thus it is our contention that the *in situ* determination of GH immunoreactivity as a function of quantity of GH being produced and proportional number of adeno-alpha-pituicytes having the ability to produce it, is a valid and even desirable procedure.

Since in this work both diminished *in situ* GH and GH-producing adeno-alpha cells were demonstrated, together with a definitive infection of the adeno-pituicytes by the BPRV; and since these findings coincided with a marked growth impairment ("thriftlessness" and wasting syndromes) and with obvious signs of thymus/spleen lympho-depletion, an at least indirect relationship between BPRV and immunodeficiency could be postulated. Such relationship would have to be explained through GH dysfunction and its effect on the thymus.

This relationship, the hypophyseal/thymic axis, had been nicely documented in at least 4 other works and approaches as cited in the most comprehensive and current treatise:[1B]

1) The possibility (in mice) to successfully treat genetically-defective naked-thymusless mice by the application of GH (Pierpaoli, 1967).
2) The possibility (in mice) to produce a wasting syndrome through the application of (rabbit) anti-bovine GH. Such a syndrome was successfully treated with bovine growth hormone replenishment (Pierpaoli, 1968).
3) The possibility to treat (in Weimaraner dogs) genetically deficient dwarf dogs with bGH with full recovery and normal maintenance of the animals (Roth, 1980 and 1984).
4) The characterization of at least another (lympho-chorio-meningitis, LCM) virus and its associated wasting syndrome as specifically infecting and affecting the GH-producing alpha-pituicytes of the hypophysis in infected animals (mice); and the possibility of treating those animals by transplanting into them a GH-producing pituitary cell line (from rats) (Oldstone), 1984).

Summary

Thirteen Holstein calves (5 controls) were case-control studied. A monoclonal antibody well characterized BPR virus (BPRV) isolate (ATCC VR-985) was inoculated intrathecally (i.t.) or intralingually (i.l.). Day by day monitoring of the following parameters was carried out through BMDP computer processing: 1) Clinical symptoms which will be described in detail; 2) Body temperatures which were unremarkable; 3) Clinical pathology data with same relevant lymphocyte changes, correlated to 4); 4) Reduced (at least 20%) dimensions (SPLDIM) and relative weight (PERATIO) of spleen and depleted immuno-configuration of lymphoid organs, thymus, spleen and lymph nodes); 5) Anti-RV neutralizing antibody (VNA) which gave mostly (5/8) negative results among the infected/affected animals even after challenging of the survivors with BPRV, one animal (1/13) gave marginal reactivity at death time and one (pre-experimental reactivity, 1/13) very high reactivity at necropsy time; 6) Presence of virus in CSF and vitreous fluids also with negative results; 7) Feed consumption/metabolic body size (MBS) ratio, only affected after 8) took place; 8) Body weight change regressions as indicators of linear growth, which plateaued ("thriftlessness") and then "collapsed"; 9) Plasma glucose levels with unremarkable results.

The BPRV was very remarkable found in lower and upper cord, cerebellum, hypothalamus. The best diagnositc specimen was the trigeminus nerve (TN) followed by the cerebellum. The hippocampus was relatively unimportant. Under regular H/E staining no Negri-bodies (NB) were detected. Immunoperoxidase-PAP (IPP) staining made NB very apparent particularly in the hypo-thalamus.

Cell bound somatotropic (STH) or growth (GH) hormone detected by *in situ* immunostaining was present in the STH producing alpha-cells in the adenohypophysis in significantly lower immunoreactive amounts in the infected/affected vs healthy animals as determined by an anti-rat STH (rSTH) staining endpoint determination ($\log_{10}$2.8 vs 3.4). The proportion of scattered STH producing alpha-cells in the adeno hypophysis was also remarkably (1 to 5 vs 25%) reduced. The difference in STH-production by infected vs uninfected cells was confirmed in an *in vitro* STH-producing rat pituitary cell culture system. An important conclusion is that rSTH and bSTH do cross-react in terms of *in situ* binding ability: anti-rGH binds only bovine STH-producing alpha-pituitary cells.

Spleen depletion was successfully studied by a (non-immune) DAB-only endogenous peroxidase stain which stained mostly erythrocytes (RBC) and by contrast allowed for an indirect determination of white vs red pulp. The former had effaced with respect to the latter particularly (but not only) around periarteriolar sheaths (T-cell area) and mantles (B-cell area). In the BPRV affected animals the RBC had invaded these two specific lymphocyte areas indicating alterations in the lymphocyte-"homing" in those areas.

Acknowledgements

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Bluetongue virus serotype 20: experimental infection of pregnant heifers

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SUMMARY: Three groups of 4 cows at 84 to 95 days, 100 to 160 days, and 170 to 180 days pregnant were inoculated both intradermally and subcutaneously with bluetongue virus serotype 20 (BTV20). Clinical observations and the viraemic and serological responses of the cows were followed for 9 to 17 weeks after inoculation.

Viraemia developed in 9 of the 12 cows and was first detected 4 to 9 days after inoculation. Viraemia was detected for 4 to 21 days and in some animals only intermittently. The titre of the viraemia was obtained in 4 cows and ranged from detectable only, to 10^{1} to $10^{2.8}$ 50% tissue culture infecting doses per ml.

Both serum neutralising and precipitating antibodies were detected in 11 of the 12 cows within 2 to 8 weeks after inoculation.

No clinical responses were seen and one cow (516) did not develop a viraemia or produce detectable antibodies to the virus. The cows, calves and foetuses were necropsied following either parturition or slaughter between 200 and 270 days of pregnancy. No virus isolations were made from a wide range of tissues from the cows, calves or foetuses and no immunoglobulins or serum neutralising antibodies were detected in the serums of pre-colostral calves or foetuses at necropsy. No gross or histopathological lesions were seen in the cows, calves or foetuses, and there was no evidence that BTV20 crossed the bovine placenta or infected the foetus.

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