Hypophysiary Involvement and Immuno/Growth Depression in Rabies I. Bovine Paralytic Rabies (BPR)

M. J. Torres-Anjel, W. Wolff, T. A. Mollett,* F. A. Martz, L. Kintner, D. C. Blenden, M. Turk, D. Volz, M. Riordan, M. Wilson, M. Stafford

Introduction

This investigation was designed to study the feasibility of extrapolating our formerly described rabies virus (RV) associated immuno- and growth-depression data from the mouse and the rat[9 11] and more recently the rabbit,[12] to the bovine model. Such information was not available from any of the classical bovine rabies studies.[1 4 6 7]

Methods

The animals. Six young Holstein bovines weighing 64 ± 14 kg were purchased from a selected farm or received from a university farm (courtesy of co-author F.A.M.) and allowed to equilibrate in our isolated unit for 9 to 17 days.

The virus. It was isolated from a typical BPR case in Trinidad (W.I.), monoclonal-antibody characterized[11] and deposited in the American Type Culture Collection (Rockville, MD USA) under the nomenclature ATCC VR-985.

Inoculation. the RV was titrated for the growth disruption effect on the rat model[9 10a11] and was injected intrathecally (i.t.) at the lumbosacral region, and/or intralingually (i.l.). The No-RV control calves were injected by either via with an equivalent volume of blank culture medium.

Virus dosage. Correcting for volume the dosages applied were consistently 10^4 ED_{50}/kg rat per calf for both the i.t. and the i.l. routes. The ratio ED_{50}/kg calf changed with each calf relative to its individual body weight (WEIGHT) since the amount of virus injected was fixed for each inoculation route.

Growth hormone. Bovine growth hormone (bGH) was radioimmunoassayed (RIA) in sequential serum samples by a technique described elsewhere.[5] Tests were performed through the courtesy of this author (co-author T.A.M.).

Confirmation. The diagnosis of RV in the affected animals was accomplished with our formerly described immunostaining technology.[9] We have already described the fluorescent (FA) as well as the immunoperoxidase-tagged (IP) antibody techniques as utilized here.[9 10 14]

Data handling. All data were stored through a terminal connected (TSO) to the UMC's main frame. Data were processed by a BMDP[3] software statistical package. Body weight percent changes, CHANGE = ((WEIGHT at day_t)/(WEIGHT at day_o) x 100) and PERCENT = CHANGE − 100. upon post-inoculation time in days were utilized, their regression as predictor of growth rate and their correlation as predictor of linearity.[10a] Other statistical parameters were also as already described.[10a] Briefly, they were the SPLDIM (spleen length x width or SPLAREA) and the PERATIO ((spleen weight)/(body weight at necropsy time) x 100).

Results

The titration. The statistical parameters for the titration of the "wasting" effect in the rat model are shown in Table 1.

The incidence. Inoculation with RV was performed in 5/6 calves and incidence of infection/affection (RV+) was 4/5.

The collapse. After either 7, 9, 14, 24 or 32 days post-inoculation (longer for higher WEIGHT at t_0), a growth rate "plateau" occurred and then a "negative growth" rate decrease ("collapse"). Both were statistically linear ($p>0.05$). The control continued to grow steadily. A summary of the statistical parameters studied is seen in Fig. 1. The statistical model also fitted (Table 2) a more recent experiment, for which no graphical representation is shown here, with very similar results.[13]

Immunostaining. the immunostaining monitoring of the RV in the CNS and trigeminal nerve (TN) of these bovines (RV+) has been presented somewhere else.[10 13]

Thymus. In the thymus of the RV+ animals there was more medulla than cortex, the former's increased area taking place at the expense of the latter's effacement. Further evidence of abnormality and immunodepression

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Departments of Veterinary Microbiology, Veterinary Medicine and Surgery, and Veterinary Medical Diagnostic Laboratory in the College of Veterinary Medicine; and USDA-ARS Animal Physiology Unit, University of Missouri, Columbia, MO 65211 USA.

**Department of Agriculture, University of Maryland-Eastern Shore, Princess Ann, MD 21853 USA (Mollett)*

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

TABLE 1. Titration (Effective Dose 50%, ED50) of the "wasting" effect in the rat model of a bovine paralytic rabies virus (BPRV) in its three "versions" associated to experimental rabies.

log ED50/kg rat injected	Effect %	Weigth at peak growth (kg)	Percen(t) Change Coefficients Regression	Correlation
No RV	0	0.138	+15.200	+0.9883
9086 (MB) Mouse brain material injected into calves (Exp. 163)				
−5.8	0	>0.100	>+22.000	>+0.9900
−4.6	50	0.080	+21.967	+0.9120
−3.5	50	0.060	+19.153	+0.9108
−2.7	100	0.050	+1.668	+0.6167
−1.5	100	0.046	+5.050	+0.7729
163 (9086) Bovine brain (calf 163.2) injected into calves (Exp. 172)				
−6.2	0	0.110	+13.052	+0.9784
−5.2	0	0.110	+13.327	+0.9770
−4.3	50	0.052	+10.365	+0.8675
−3.2	100	0.055	4.350	0.7489
−2.5	100	0.049	3.317	0.6720
−1.3	100	0.048	1.153	0.3098
F163 (9086) Tissue culture (1 mouse, 1 bovine, 3 BHK-21) utilized for challenge (Epx 172)				
−4.52	33	0.110	+18.887	+0.9715
−3.47	50	0.087	+13.021	+0.7579
−2.48	67	0.093	+11.007	+0.7994
		0.068		
−1.51	100	0.034	−0.425	−0.4245
		0.043		
		0.048		
−0.51	100	0.046	−0.589	−0.1702
		0.036		
		0.036		

* The effect (affect/total animals) is measured in terms of "wasting" (immuno and growth depressions) with eventual death).

was the presence of many "punched" areas (solutions of continuity) indicating recent necrosis of thymocytes which left typically unstained spaces. The latter phenomenon is better described as the "starry sky" phenomenon. Macroscopically, the thymus of the RV+ calves had undergone considerable involution with no thymus seen in the right side of the chest and a maximum of about 20 to 30 g of thymus left in the left side of the thoracic cavity. Cervical thymus was minimal or inexistent. Because of the thymus involution, the collection of samples from this organ was scarcely sufficient for histopathology. The lobules were reduced in size because of a reduced number of thymocytes in the cortex. The cortex was about ¼ to ½ the diameter of a high power field. A final diagnosis summary would be untimely involution, effacement of the cortex, and individual thymocyte (T cell) area necrosis (Fig. 2) in the RV+. Thymus appearance in the healthy No RV controls was perfectly normal (Fig. 2).

Spleen. Although the changes in the bovine spleen topography are not of the exuberancy of the rat's spleen[9 11] topographical details in the RV+ animals included depletion both in mantle (B cells) and (but mostly) periarterialar

FIGURE 1. The linear regression/correlation model for normal, pre- and post rabies virus + collapse.
Round open circles = uninoculated No rabies virus healthy control (Calf 1)
Round open squares = Rabies virus + infected/affected (Calf 2)
Dark circles = Rabies virus + infected/affected (Calf 3)

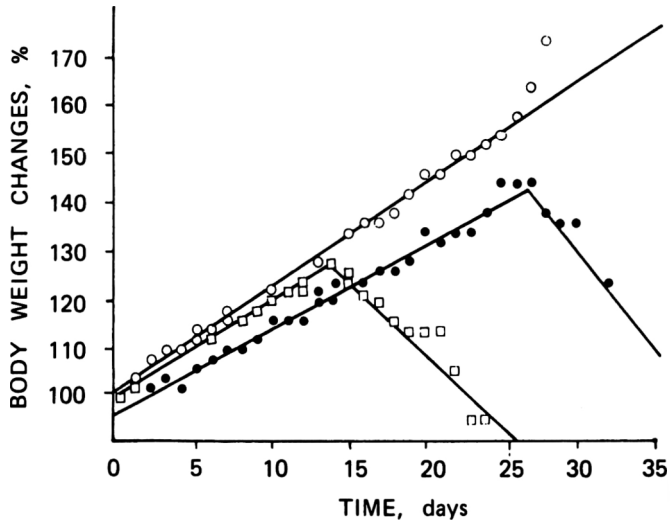


TABLE 2. Statistical analysis of linear body weight change patterns as affected by a bovine paralytic rabies virus inoculated (10 4.0 ED 50/kg rat) intrathecally (numbers) or intralingually (letters) in (60 ± 12 kg) weanling bovines.

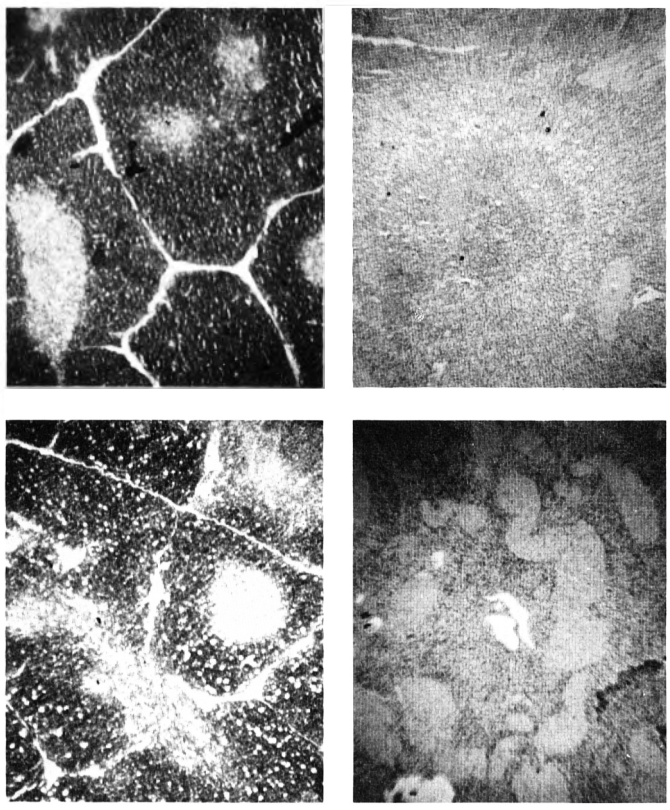
Calf No. (weight kg)	Post-inocul. Day of Maximum Weight	Portion of Curve	Coefficients Regression*	Correlation**
1 and C	Last***	Positive growth	+2.2503	+0.9885
(No virus)		....	+1.4120	+0.9970
(41)				
(70)				
2 and B	24	Positive growth	+1.5929	+0.9739
(53)	7		+1.2647	+0.9870
(70)				
		Collapse	−3.2788	−0.9538
			−4.4000	−0.9680
3 and A	33	Positive growth	+1.5954	+0.9934
			+1.5123	+0.9870
(59) 9				
(69)		Collapse	−2.6229	−0.9293
			−1.9643	−0.9600
Pool		Positive growth	+1.6750	+0.9220
			+1.3832	+0.9730
		Collapse	−3.0665	−0.9467

* Body weight changes, % (Y), times (X).
** Body weights (kg) and body weight changes, %; the two types of correlation coefficients were identical within three decimal places.
*** Control (No RV) animals increased in body Weight throughout the experiments.

lymphatic sheath (T cells), friability and solutions of continuity indicating immunodepression. In the RV+ animals the germinal centers appears relatively small and inactive and the parafollicular area appeared to have been infiltrated with neutrophils. In the RV-collapsed animals, a visible upsurge of connective vs pulpar tissue was evident thus corroborating a depletion of the latter (Fig. 2). Spleen microtopography in the healthy No RV controls was perfectly normal.

Both the thymus and spleen data have been confirmed in a more recent set of experiments[13] and by a different pathologist-consultant (courtesy of Dr. M. Turk).

FIGURE 2. Immunostatus of linearly growing calves uninfected (No RV) and infected (RV+) with bovine paralytic rabies virus as estimated by lymphoid organ topography (1 cm = 84 &m) (119x).
Left: Thymus; Right: Spleen.
Up: No RV; Down: RV+
For interpretations see text.



The hypophysis (pituitary gland). The evidence of RV+ infection in the bovine neuro- (posterior) hypophysis and adeno- (anterior) hypophysis had been illustrated elsewhere.[10 11] This time we selected to also illustrate the results of RV+ infection with respect to the pars intermedia in particular (Fig. 3). The adeno-hypophysis RV+ infection though is also shown in Fig. 4. All (5/5) RV infected/affected animals presented RV+ hypophysiary infection.

The IP analysis [9 11] of the adeno-hypophysiary tissue revealed cells that took up the anti-Rv stain appeared larger than the adjacent chromophobe, acidophil and basophil cells. Because of the inherent contrast limitations of black and white photography, no pictures are shown of the IP staining of hypophysis. It may be said though that 15 or 20 isolated large cells widely scattered in the adeno-hypophysis contained RV antigen granules in the cytoplasm. These cells appeared to be larger than the adjacent chromophobe, acidophil and basophil cells. These stained cells could not, but are in the process of, being identified. No obvious RV antigen was detected in the neutral hypophysis by IP staining, but on the contrary FA staining was relatively rich.[10]

When evaluated by H/E the adenomatous portion of the pituitary was mostly normal. The pars nervosa contained three or four glial nodules. There was a mild perivascular cuffing and/or glial nodules indicating some inflammatory reaction.

Growth Hormone. Table 3 shows the statistical analysis of a regression of growth hormone upon the percentage body weight reduction (PERRED) as shown graphically in Fig. 5. As it may be seen the values about the mean were very much more stable in the healthy calf than in the two RV-affected ones. Both the regression coefficient and the correlation (expressed as correlation to the square power, or R^2) were also higher in the affected animals indicating not only a closer relationship between body weight changes and growth hormone but also a more defined trend. This is also shown by the performance of a very simple non-parametric test comparing the separation of data items (above vs under) by the regression line, a test which was also indicative of higher "oscillation" and broader spreading of the GH-data of the affected vs control animals. The separation in the healthy animal was 7 vs 6, while it was 4 vs 7, and 5 vs 9 respectively in the RV+ animals.

Discussion

Growth hormone. It is possible to produce a wasting phenomenon in the absence of any virus, indistinguishable from our RV-induced collapse using either the rat or the bovine model, by injecting animals with an anti-bovine (rabbit produced) somatotropin.[8 12] Such anti-hormone treatment induced changes in body weight, spleen size, and thymus and spleen topographies that were remarkably similar to ours in terms of chronology, kinetics, and microscopic appearance. Thus, the finding of hypophyseal RV-infection as presented here and before[9 10 11 13] may assist in providing a link between decreased levels of growth hormone and other endocrine dysfunctions that would explain the high pathogenicity and lethality of bovine rabies. This could be more important if the remarkable and consistent RV+ hypothalamus infection[13] is also brought into the picture. Hypothalamic RV+ infection has been very frequently described in the classical RV literature although with no previous attempt to derive any hormonal or

immunological conclusions or interpretations from it.

Growth hormone release into the blood stream is a well characterized pulsating phenomenon[5] and of course detailed correspondence throughout a period of time requires complex and repetitive determinations in multiple samples. Such an effort we are pursuing at the moment[13] which has also been useful in disassociating the growth plateauing (primary wasting) from the anorexia and/or the glucose plasma levels.[10a12 13] Although no definitive information on levels of GH is still definitive, within the limited framework of this experiment some general observations become obvious. There was a different trend in the affected vs healthy control animals as determined by a simplified regression analysis. The GH determinations in the RV-infected/affected animals showed a broader variation (larger standard deviation), a more definitive trend (larger regression coefficient) and a tendency to follow the changes in body weight reductions as estimates of "negative" growth. There is an enormous dilution factor inherent to GH determinations in either plasma or serum. A ratio of ng to liter is involved, that is a 10^{-12} or higher dilution factor. This

FIGURE 3. Infection of the hypophysiary pars intermedia by rabies virus (RV) in young bovines.

Up: Florescent (non-immuno) microscopy of the bovine pituitary gland or hypophysis. Note the perfect delimitation between the neurohypopysis, the pars intermedia (intermediate lobe) and the adeno-hypophysis. The neuro- and the intermediate are clearly separated by the cleft. The intermediate hypophysis has a "branching" appearance (1 cm = 84 nm) (119x).

Left: (Non-immuno) flourescent microscopy Right: Hematoxylin/Eosin stain

Down: Anti-RV immunofluorescence showing particularly RV-infection of the intermediate hypophysis. The horn that projects towards the bottom in the lower left and the hump that projects toward the right in the right picture are the same ones seen in the upper left picture but at a lower magnification. In this case the FITC filter system has been implemented for actual immunofluorescence microscopy. Note the specific and particular presence of RV in some large cells. (1 cm = 29.4 u) (340x).

For interpretation see text.

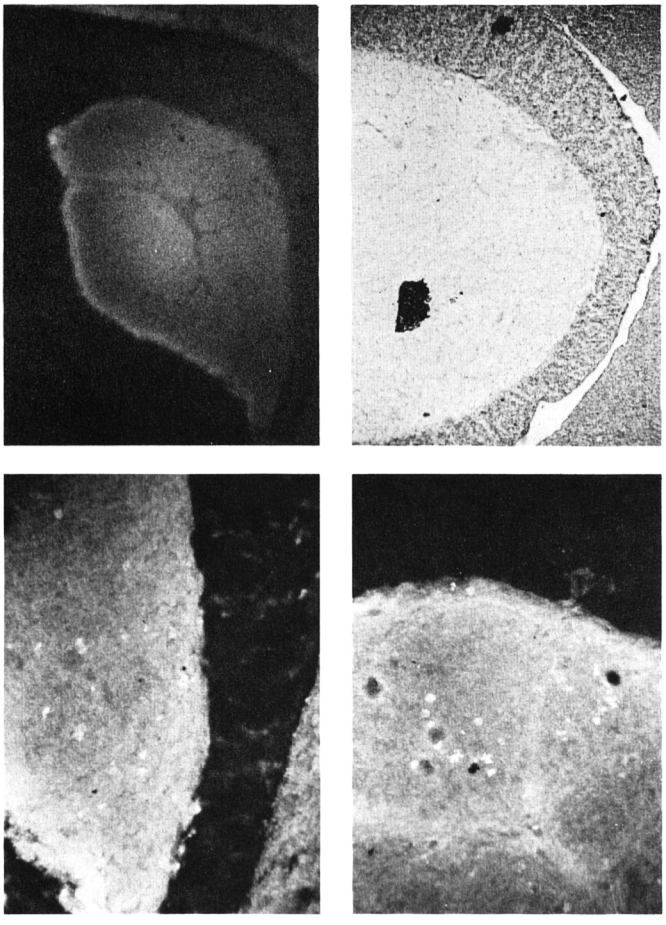


FIGURE 4. Immunofluorescence confirmation of Rabies Virus + infection in the bovine adenohypophysis. Note the massive multiple "minute" type of infection of the adenohypophysiary glandular tissue that involves approximately 50% of the cells. This "saw dust" appearance of the RV-antigen is typically seen when RV invades non-neuronal cells in which it is probably less "comfortable" (1 cm = 29.4 u m) (340x).

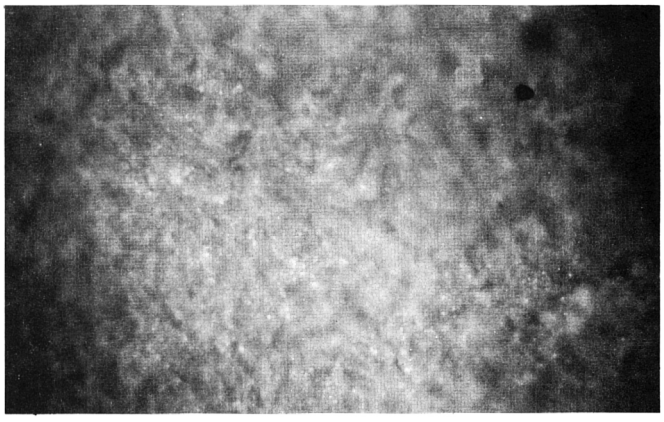


TABLE 3. Regression analysis of growth hormone (GH) upon percentage body weight reduction (PERRED-upper) or post inoculation day (lower) in linearly growing calves during rabies virus (RV) infection/affection.

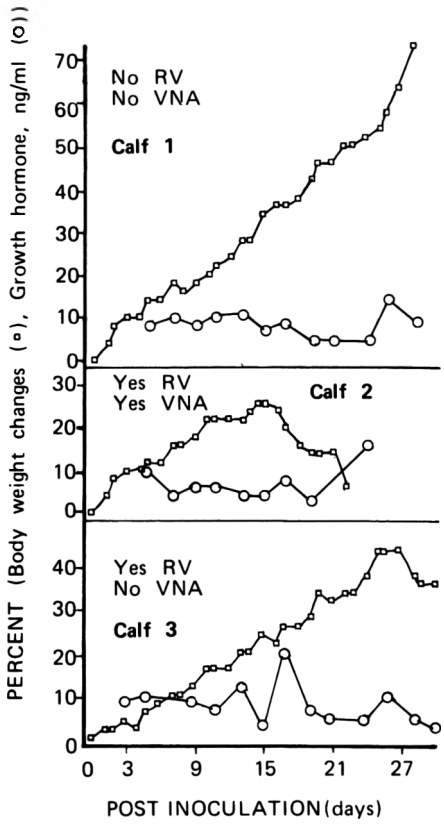
Calf No. (RV Status)	Mean (± SD)	Regression Coeff.	Separation * over	under	Correlation Coeff. (R^2)
1	8.20	−0.0384	7	6	0.0646
(No virus)	(2.91)	−0.0989			0.0762
2	7.49	−0.3618	4	7	0.2285
	(4.61)	+0.24237			0.1276
3	7.45	−0.1171	5	9	0.1076
	(4.63)	−0.2040			0.1481

* Observed number of data items remaining over and under the statistically drawn regression line. Expected are approximate equal numbers. Chi-square.

FIGURE 5. Body weight changes and growth hormone monitoring in linearly growing calves inoculated with bovine paralytic rabies virus (RV).

a) Body weight percent changes (clear squares) regressions during positive growth (+ 1.60 ± 0.34) and during collapse (−3.0 ± 1.0) were significantly positive or negative (different from 0) at p 0.001; regressions were 92% (Table 2).

b) Growth hormone (clear circles) "oscillations" were statistically and significantly (p 0.05) different between the No RV control and the infected/affected animals; among these, Calf 2 was particularly outstanding (Table 3).



dilution may result in some difficulties in interpretation particularly when, as is the case with rabies, dehydration may be involved. Perhaps a better approach would be to look at GH determinations in the adeno-hypophysiary alpha cell that produce it, by quantitative immunostaining. Such an approach has been taken[12 13] and the results corroborate GH alterations in the RV+ animals.

Growth. Linearity, stoppage, plateauing and collapse followed an almost identical kinetics in the bovine as in the rat[9 10a 11] model although of course the magnitude of the dimensions was different. The plateauing-collapse was related to either RV dosage (variable ED_{50} in the rat) or WEIGHT at t_0 (variable in the bovine), either way with an earlier collapse in the higher dose/weight ratio and with a steeper collapse (<-B) in the delayed appearance of the phenomenon. This suggested a cumulative-threshold type of phenomenon.

The growth impairment approach in the study of BPR, with its hormonal-immunological consequences is not only important but also original. Former studies had utilized either heavier (mean 210 kg) animals,[4] or older[7] (2 to 9 years); or, even when younger[6] (4, 15 and 90 days of age), the study was done without any concern with respect to growth, body weight or immunological organs. Actually, studies of the reticuloendothelial system with respect to rabies were until now a rarity in all species, and completely absent in BPR.

Immunopathology. A very marked immunodepletion developed in the RV+ animals as evaluated by macroscopic evaluation (SPLDIM, PERATIO, thymus-cervical disappearance, thoracic diminution) and microscopic histotopography. The most dramatic results[9 10a 11] had been seen first in the rats spleen: SPLDIM and PERATIO reduced to 2/3 the healthy ratio, effacements of white pulp (vs red), periarterioral sheath a T cell area; and second in the bovine thymus: marked effacement of cortex, corresponding increase in size of medulla, loss of cellular density all throughout the organ and the "starry sky" phenomenon: "punched" areas indicating thymocyte necrosis and loss of continuity. Although each of these target organs was the outstanding site in either species, the phenomenon occurred in both organs and in both species in varied but uniform degrees with all other lymphoid organs affected: lymph nodes, peyer patches, etc.

Hypophysiary/hypothalamic involvement. In the bovine the anterior, the posterior and the pars intermedia portions of the hypophysis were consistently infected with RV, with tendency for infection to be particular to some specific cells scattered among chromophobe, acido- and basophils, and being evaluated at present. The hypothalamus was consistently infected. We seem to be able to detect 10x to 100x more RV antigen by FA as compared to IP.[12] Such discriminant ability makes IP into a very selective and conservative test which detects areas with particularly high content of RV+ immunoreactivity. It also makes the conegativity (specificity) of IP very high.[14]

We consider the discovery of the hypophysiary infection as the most remarkable contribution discovery of our work, particularly since it was seen in conjuction with growth impairment and immunodepletion. The infection of the posterior hypophysis, although perhaps a less unexpected result since it is of diencephalic and thus neurological origin, is still remarkable. It would seem indicative that a dysfunction in its hormone vassopressin, an antidiuretic principle, might be involved in the observed dehydration, a consistent symptom throughout our studies, not explainable in terms of "hydrophobia" or thirst alone.[12] The infection of the anterior hypophysis is of course the most pertinent observation in order for us to be able to explain not only growth impairment but also immunodepression of central origin. The infection of the pars intermedia is the

observation that we emphasize the most in this work since it not only may have consequences *per se* and in the production of its own hormones; but also because of its prominent relative size and importance in the bovine species where it seems to be (exceptionally) involved in the synthesis, production, transport and/or management of GH itself.[2] This is confirmed by our own anti-growth hormone IP *in situ* staining technique.[12 13]

Growth. The kinetics of the titration of the BPRV wasting effect have been shown profusely somewhere else.[9 10a 11] Suffice it to say that the wasting effect is calculated not only in terms of Effective Dose 50% (ED_{50}) but also in terms of the maximum weight at which linear growth stops and collapse starts. The higher effective dilutions gave an accelerated appearance of the phenomenon although it is steeper at the lower effective dilutions or other-than-i.c. inoculation. Based on this concept it was important to evaluate an ED_{50} base on the immuno-growth depression effect. Innovatively we calculated it and based it on the maximum WEIGHT reached at the plateauing of the growth curve and before the collapse.

Rabies as a disease has been recently shown to be not necessarily inexorable in many species anymore including man. Mortality however continues for practical purposes to be extremely high (>99%). Lethality causation remains as one of the best kept secrets in nature, in spite of RV being one of the best and most studied viruses. There is a very outstanding gap between the unexplained and extremely high lethality, and the recent and sophisticated developments on RV studies: i) monoclonal antibody characterization of strains; ii) genome and epitope characterization; iii) amino acid sequencing of latter; iv) characterization of specific glycoprotein sites associated to pathogenicity and/or virus antibody neutralization; and v) more recently, genetically engineered vaccinia-virus "vectoring" of RV genome with promising RV immunogenicity against the vectoree (RV). The characteristic but relatively mild encephalomeningitis seen in rabies does not explain death to satisfaction. (Actually in our experiments with Fully Pathogenic (FPV) vs Less Pathogenic (LPV) variants of RV we have detected a much higher level of encephalomeningitis in the animals infected with LPV among which the survivability is remarkably higher by definition.[9] In other words, the more dramatic the encephalomeningitis the higher the surviving rates. Under those conditions, we use a marked encephalomyelitis as a predictor of survival). Also, humoral circulating antibody does not necessarily correspond to actual immunity and interferon is not absolutely related to survival; the latter two however are the only two accepted predictors, as deficient as they may be, of immunity and the only two available principles with some therapeutical application at present.

A marked impairment of the growth mechanisms with hypothalamic/hypophysiary/thymic involvement and with immunodepletion clearly points towards a marked central hormonal dysfunction probably involving not only growth hormone (GH) but other hormones as well. It is important to remember that GH is the master hormone for not only growth (protein synthesis), but also other important functions including mobilization of essential ions (Na+, K+, Cl–), phosphorus, glucose, fat, ketones, etc. Also, GH is one of the most recognized master hormones for the development, function and physiological (with age and immuno-maturity) involution of the thymus. The production of GH is hypophysiary but the regulation is hypothalamic (as for other pituitary hormones) via GH-releasing factor (GRF) and GH-inhibitory factor (GIF) or somatostatin.

It was traditional to assume that the RV-associated emaciation reported by other authors was typically the result of secondary malnutrition, an almost-by-definition consequence of rabies in which reaching for food (accessing), grasping (avidity), and swallowing (deglutition) were impaired due to paralysis of different sets of motor muscles due to the infection motor craneal pairs ("hydrophobia," etc.). Our recent studies[9 10a13] showed that it was true that secondary malnutrition was of course an important contributor to rabies final fate but that a primary "wasting" was chronologically, kinetically, statistically, biologically and immunologically independent of RV-secondary malnutrition, anteceded it and preceded any noticeable changes in either feed or water consumption. Such results have been corroborated here and in other of our works[10a12 13] in the bovine model. Towards the end of the life of the RV+ animal, of course, the two phenomena (primary "wasting" and secondary malnutrition) were intermingled and synergistically contributed to the fatality.

The biological consequences of this newer hypophysiary/nutritional approach in terms of "herd" health, population dynamics, infection susceptibility and immunity are obviously daring, fascinating and provocative in terms of bovine endemic and/or epidemic rabies. Also the possibilities of hormonal/nutritional manipulation with respect to mass or individual anti-RV vaccination management are also interesting. Finally the possible consequences of vaccination with live "attenuated" less pathogenic virus (LPV) strains as used today in terms of growth (and thus milk and beef production), at-large immunocompetency and consequences in bovine nutrition, and the possible interrelation of RV AND RV- vaccination with other pathogens has to be considered. The LPV strains have been shown to also produce immunodeficiency (significant although reversible) and growth depression (also reversible) in the experimental rat and mouse RV= infection surviving models.[9]

Summary

Linearly growing bovines were inoculated with a bovine paralytic (BP) rabies virus (RV) isolate. The control animal continued growing steadily. The collapse rate (-3.0 ± 1.0) was higher (1.8x) than positive growth rate ($+1.6 \pm 0.34$) and

overall reduction in growth was $\leq -4.5\%$ to reach its steady state and to go through and remain significantly different ($p<0.001$) from 0. Growth affection coincided with deviations from the expected "oscillation" ranges in the growth hormone (GH) levels in the serum. The hypothalamus, neuro- (posterior) and adeno- (anterior) hypophysis and also the intermediate lobe, were consistently infected with RV antigen distributing itself with predilection for some particular cells. Thymus involution and thymocyte necrosis of the "starry sky" type, spleen depletion of the periarteriolar sheath and lymph node depletion of typical areas indicated a predominantly T cell depletion. Four elements strongly suggested central hormonal/nutritional involvement in rabies associated with "wasting syndrome": i) hypothalamic infection, ii) hypophysiary infection, iii) dramatic growth affection, and iv) immunodepression. We postulate this mechanism to take place through growth hormone (and other hormones) dysfunctions. Food and water deprivation's influence on the phenomenon was secondary, came late and independently from the primary phenomenon, although it also became synergistic. Rabies, thus is an interesting model for "wasting syndrome," both primary and secondary, as associated to immunodeficiency diseases, human AIDS' included.

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