Public Health

WHICH ANTI-HTLV III/LAV ASSAYS FOR SCREENING AND CONFIRMATORY TESTING?

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Summary In preparation for routine anti-HTLV-III/LAV testing in the UK five commercial assays (A–E) were evaluated using 360 sera selected on clinical and epidemiological grounds. These comprised 220 specimens from blood donors, 83 specimens from patients in high-risk groups, and 57 specimens with features likely to produce false-positive results. Probably erroneous positive results arose from assay A in all three categories and assay B in the second and third categories. These reactions were much more common after specimens had been heated to 56°C for 30 min. Except that an anti-HLA DR4,B5-containing serum was repeatedly positive by C, assays C, D, and E apparently did not give rise to false-positive results. Results by these three assays were also highly reproducible. In tests on serum dilutions the highest titres were obtained by assays A and D, but assays C and E discriminated most clearly between anti-HTLV-III/LAV positive and negative sera. These two assays were rapid and convenient and seemed particularly suitable for testing blood donations. Assay D was almost comparable with them in performance but more difficult to use. The commercial assays C, D and E, an antibody capture assay, and a simple immunofluorescence test could be the basis for a methodologically diverse national system of primary and confirmatory testing for anti-HTLV-III/LAV.

INTRODUCTION

THE first assays for antibody to human T-lymphotropic virus type III/lymphadenopathy-associated virus (anti-HTLV-III/LAV) were introduced during 1984[1,2] and the first commercial assays were licensed for blood donation screening by the US Food and Drug Administration (FDA) earlier this year. There had been suggestions of a high frequency of false-positive results in these assays,[3] and the UK Departments of Health decided that the commercial assays should be evaluated before any were recommended for use in British blood transfusion or clinical laboratories. Assessments of the available kits were made in June and July, 1985.

MATERIALS AND METHODS

Sera

Three categories of serum specimen—blood donor (BD), high-risk group (HRG), and potentially false-positive (PFP)—were assembled in ignorance of anti-HTLV-III/LAV reactivity. 220 were from successive donors at the North London Blood Transfusion Centre; 83 were from HRG individuals (4 with AIDS, 37 with persistent generalised lymphadenopathy, 11 contacts of AIDS or of persistent generalised lymphadenopathy, and 31 haemophiliacs receiving commercial factor VIII concentrate); 57 were from patients whose sera might give rise to false-positive reactions (12 rheumatoid factor positive, 5 Paul-Bunnell positive, 14 with other acute viral infections, 6 with haematopoietic malignancies, 8 with single species autoantibody, 4 with antibody to particular HLA types,[4] and 8 with panreactive anti-lymphocyte antibody). Dilutions from 1 in 50 to 1 in 800 of 4 sera previously found to be anti-HTLV-III/LAV positive were made in negative human serum. Specimens and serum dilutions were each divided into 15 equal parts and stored in sets in separate containers at −30°C.

Type I Ag + specimen + anti-human Ig-enz + substrate (A, B, C, D)

Type II Ag + { specimen / anti-HTLV III/LAV-enz* } + substrate (E, COMPRIA)

Type III Anti-γ + specimen + Ag + anti-HTLV III/LAV-^{125}I (GACRIA)

Fig 1—Three types of solid phase assay for anti-HTLV-III/LAV.

*^{125}I for COMPRIA.

Commercial Assays

Five commercial enzyme-linked immunosorbent assay (ELISA) kits for anti-HTLV-III/LAV were evaluated and, for comparison, a set of specimens was examined by a competitive radioimmunoassay (COMPRIA)[5] and an IgG capture assay (GACRIA) developed in the Virus Reference Laboratory, Colindale. The assays were of three types (fig 1). Commercial assays A–D (type I) and E (type II) used various forms of polystyrene solid phase (assay A 6 mm beads, B whole microtitre plates, C 12-well microtitre strips, D 4 mm coated ferrous beads, E double 8-well microtitre strips). COMPRIA was also a type II assay. GACRIA (type III) used polystyrene beads coated with antibody to the γ chain of human IgG; with intervening washes these were incubated with 1 in 50 dilutions of the specimens, with a lysate from HT/H9/3 cells (cell line supplied by Dr R. C. Gallo), and with ^{125}I-labelled IgG prepared from a serum with a high anti-HTLV-III/LAV titre. As far as is known the other assays were also based on antigen derived from the HT/H9/3 line except for assay E for which antigen from the CEM-CBL 1 line was used.

In assays A–E results were expressed as optical density (OD) values and related to a cut-off value calculated from control OD values according to the manufacturers' instructions. In assays A–D OD values above cut-off and in assay E OD values below cut-off indicated positivity. COMPRIA results were expressed as percentage inhibition of ^{125}I binding and GACRIA results as test:negative ratios, both with reference to the mean negative control value.

Sufficient kits for 1100 tests were purchased for the evaluation of each commercial assay, and washers and ELISA readers were rented as necessary. Manufacturers' representatives trained the three operators until satisfied that they were competent. For each evaluation a new set of stored samples was thawed and tested, according to the manufacturers' instructions, in four phases: (1) all specimens including duplicate tests of the serum dilutions, (2) repeat testing of all HRG and other reactive specimens, (3) after heat treatment at 56°C for 30 min, all specimens as in (1), and (4) repeat testing of heat-treated specimens as in (2). Tests on heated sera were included because this treatment is often used in clinical laboratories to make high-risk specimens safe.

The evaluations were done in the order A, B, E, COMPRIA, GACRIA, C. D. The OD values and qualitative results were entered on a data base ('Datamaster') and analysed.

RESULTS

Specificity and Reproducibility

Table I gives the results and fig 2 illustrates the distribution of values for unheated specimens (phase 1) by each commercial assay. In all assays except A, which had 17, there were 5 or fewer values within two intervals of the cut-off. 11 HRG specimens were anti-HTLV-III/LAV negative by all assays, and in the interpretation of the results it is assumed that these 11 and all the BD and PFP specimens were negative and that the remaining 72 HRG specimens were the only true positive ones in the evaluation set. OD values above cut-off

TABLE I—RESULTS ON TESTING UNHEATED SPECIMENS FOR ANTI-HTLV-III/LAV (PHASE 1)

Group	Assay result	Assay						
		A	B	C	D	E	COMPRIA	GACRIA
Blood donors (n = 220)	Positive	1 (1†)	0	0	0	0	0	0
	Negative	218	220	220	220	220	220	220
High-risk group (n = 83)	Positive	72	72	72	71	71	72	72
	Negative	11	11	11	12	12	11	11
Potential false positive (n = 57)	Positive	11 (4†)	4	1	0	0	0	(1†)
	Negative	42	53	56	57	57	57	56

*Manufacturers are: A = Abbott, B = ENI, C = Organon, D = Ortho/Litton, E = Wellcome. COMPRIA and GACRIA are Virus Reference Laboratory assays.
†Equivocal.

arose from 15 negative specimens by A, 4 by B, and 1 by C. Most of these were PFP specimens, and all the commercial assays except C gave on average stronger reactions with these than with BD specimens, even though the results on them were largely negative. After heat treatment (phase 3) assay A gave 266, B 82, D 12, and the remainder fewer than 4 values within two intervals of the cut-off (not shown). There were 250 values for negative specimens by A, 48 by B, and 1 by C that exceeded the cut-off. Since only 15 heat-treated specimens gave negative reactions phase 3 results by assay A are not analysed further.

On repeat testing (phases 2 and 4) considerable variation arose in OD and RIA values though changes in result were uncommon except by assays A and B. Before heat treatment by assay A there were 3 changes from positive to negative, 4 from positive to equivocal, and 5 from equivocal to negative. By assays B–E, COMPRIA, and GACRIA there were no changes or only 1. Repeat testing after heat treatment by assay

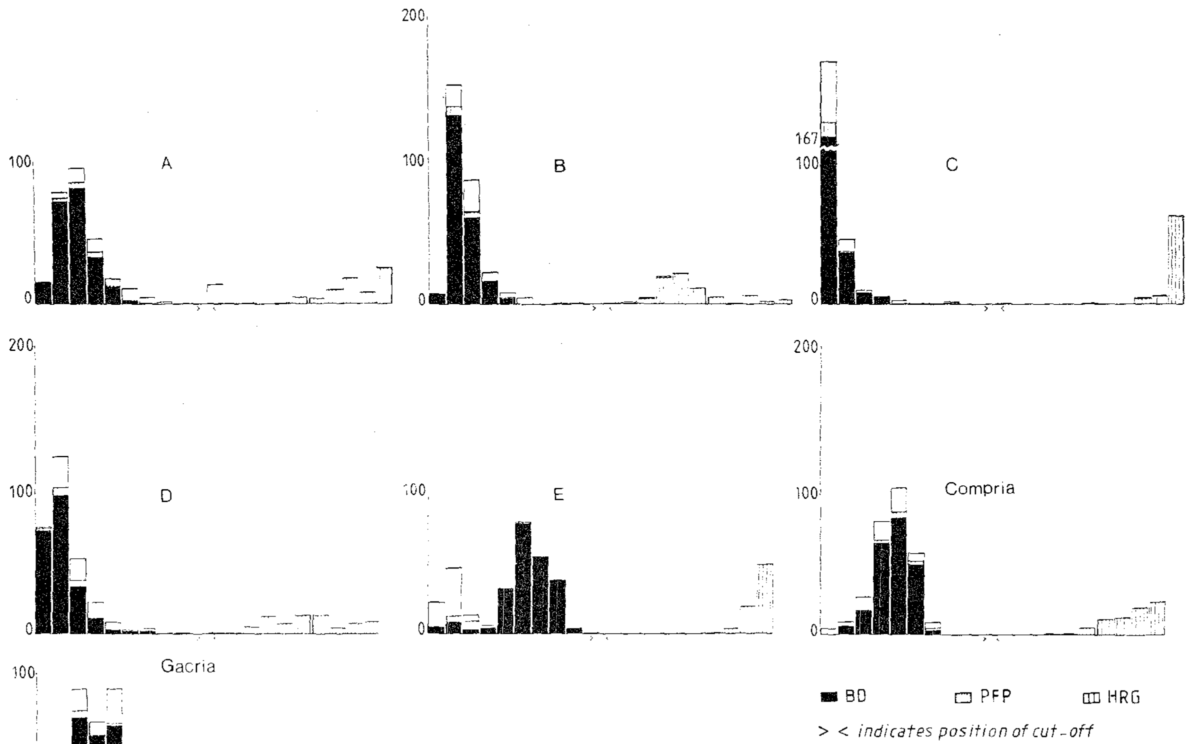

Fig 2—Distribution of positive and negative values in phase 1 for seven anti-HTLV-III/LAV assays.

Ranges from minimum value to cut-off and from cut-off to maximum value have both been divided into ten equal intervals. Number of values in each interval for three categories of specimen are displayed OD values above 2 have been counted into a 21st category. In assay E, for which OD values below cut-off were positive, intervals are in reverse order.

Cut-offs and minimum and maximum values are:

Assay	*Min*	*Cut-off*	*Max*
A	0·013	0·114	>2
B	−0·01	0·100	>2
C	0·065	0·489	>2
D	0·078	0·406	1·629
E	0·102	0·514	1·650
COMPRIA	−29	37·5	98
GACRIA	0·10	3·00	60·36

TABLE II—DISCREPANT ANTI-HTLV-III/LAV RESULTS ON 15 POTENTIALLY FALSE-POSITIVE SPECIMENS

Specimen	Assay*			
	A	B	C	GACRIA
Anti-hepatitis-A IgM	E (−)†	—	—	—
Anti-hepatitis-B core IgM	E(−)	—	—	—
Paul-Bunnell positive	+ (+)	—	—	—
Chronic lymphatic leukaemia	E (−)	+ (+)	—	E (−)
Anti-HLA DR4, B5	+ (+)	+ (+)	+ (+)	—
Pan-reactive antibody	+ (+)	+ (+)	—	—
Pan-reactive antibody	+ (E)	—	—	—
Pan-reactive antibody	+ (+)	+ (+)	—	—
Pan-reactive antibody	E (−)	—	—	—
Anti-thyroid-microsomal	+ (E)	—	—	—
Antinuclear	+ (−)	—	—	—
Anti-smooth-muscle	+ (+)	—	—	—
Anti-parietal-cell	+ (−)	—	—	—
Anti-liver/kidney-microsomal	+ (E)	—	—	—
Anti-mitochondrial	+ (E)	—	—	—

*All 15 specimens were negative by assays D, E, and COMPRIA. Remaining 42 PFP specimens were negative by all assays.
†Repeat results in parentheses. E = equivocal.

TABLE III—TITRES OF FOUR ANTI-HTLV-III/LAV POSITIVE SERA

Serum	Assay						
	A	B	C	D	E	COMPRIA	GACRIA
1	>8*	2	2	>8	4	>8	2
2	4	2	1	4	4	>8	1
3	4	1	1	4	2	>8	2
4	4	2	1	4	4	>8	2

*Dilutions to 8×10^2 tested in duplicate. The titre is the last mean value greater than cut-off.

B gave 29 changes from positive to negative and 2 from negative to positive. By the other assays (except A for which only a batch of 30 specimens was re-tested) there was never more than 1 change.

Discrepancies between assays arose in all three groups: before heat treatment all *blood donor specimens* were anti-HTLV-III/LAV negative by all assays except for 1 weakly positive and 1 equivocal result by A (both negative on repeat). With assay A results excluded, all BD specimens were also negative after heat treatment except 21 positive by B and 1 equivocal by COMPRIA.

Before heat treatment 70 *high risk group specimens* were anti-HTLV-III/LAV positive by all assays, 11 were negative by all assays, and 2 others were each negative by one assay (D and E) (both were positive on repeat). After heating the same 11 specimens were negative by all assays (A excluded) except for 2 positive by B which were negative on repeat. The remaining 72 HRG specimens were positive by all assays except for 1 that was GACRIA negative (on repeat strongly positive).

For *potentially false-positive specimens* few discrepancies arose before heat treatment except by assay A (table II). After heating (excluding A) all PFP specimens were negative by D, E, COMPRIA and GACRIA, but 25 were positive by B and 1 by C.

Sensitivity

The sensitivity of the commercial assays was assessed by titrating 4 anti-HTLV-III/LAV positive sera. Before heat treatment titres were in the range 400 to above 800 by assays A, D and COMPRIA, 200–400 by assay E, and 100–200 by assays B, C, and GACRIA (table III). After heat treatment titres were the same as before by assays C and COMPRIA, within one dilution by E, two dilutions by D and GACRIA, and three dilutions by B. The distribution of the values for the 72 positive HRG specimens was another indication of sensitivity (fig 2). In assays C and E at least 68(94%) of these specimens fell in the top three intervals both before and after heat treatment.

Convenience

The number of manipulations in each commercial assay, the time taken to complete it, and the cumulative score given on five criteria by three operators are shown in table IV. Considerable difficulty was encountered in the supply of kits for assay B, and some replacement kits for assays A and E were substituted by agreement between the manufacturer and the evaluators. Operators noted undesirable features in some rented equipment, such as spraying or flooding during washing, splashes during reagent addition, and poor legibility in the print-out of a reader.

DISCUSSION

An anti-HTLV-III/LAV assay needs to be both sensitive and specific. These qualities are not easily combined, but the aim should be for reliable assays that have both. The commercial kits currently in use have been devised primarily for screening blood donations and the emphasis has been placed on sensitivity, but this carries with it a risk of loss of specificity. Some of the assay kits gave false-positive reactions (mostly weak) in this study, particularly on PFP specimens. Specimens of this sort are often said to be "sticky", and the same effect may be produced in any serum by heat treatment. After heat treatment in these evaluations there were many erroneously positive reactions by assays A and B for all categories of specimen. Assays D and E also performed differently after serum had been heated (data not shown), though this did not lead to false-positive results. Assay C gave rise to a false positive result on a single specimen with strong anti-HLA DR4, B5 both before and after heat treatment. It therefore seems that, though this study and a large American survey[6] have found a low false-positive rate in unheated blood donor specimens, some categories of clinical specimen, many heat-treated specimens, and a few specimens with HLA antibodies give uncertain results in some commercial enzyme

TABLE IV—CONVENIENCE AND ACCEPTABILITY OF ASSAYS FOR ANTI-HTLV-III/LAV

Criterion	Assay						
	A	B	C	D	E	COMPRIA	GACRIA
No of stages*	8	8	8	7	5	4	8
Total time (h)†	$5\frac{1}{2}$	$2\frac{1}{2}$	$2\frac{3}{4}$	$3\frac{1}{4}$	2	$17\frac{3}{4}$	$23\frac{1}{4}$
Total score (max 60)‡	41	32	48	29	42	ND	ND

*Specimen predilution, plate loading, incubations, washing, colour development, reading.
†Estimated time to obtain results on 90 specimens with controls.
‡Three operators gave up to 4 points for each of five features: training given, clarity of instructions, ease of use of equipment and of reagents, and simplicity of test procedure. ND = not done.

assays. In particular, heat treatment introduces a risk of error,[7] and we suggest that any positive finding on a treated specimen should be checked by testing an unheated sample.

On repeat testing several inconsistent findings which may have been due to machine or operator failures were resolved. The 2 false-negative results that were recognised in the evaluation seem attributable to such causes; both became positive on repeat. Other inconsistencies, some persisting on repeat testing, were probably due to faults intrinsic to the assays. Although the values for many of the specimens giving unstable results were close to the cut-off, an equivocal range was only defined in one commercial assay (A, as part of its spectrophotometer programme). We think that all borderline specimens, negative as well as positive, should be re-tested and suggest that, if necessary, the user himself should set the limits for an equivocal range of values.

Assays A and D appeared to be the most sensitive commercial assays by titration, and assays C and E discriminated most clearly between the 72 specimens in the evaluation set assumed to be anti-HTLV-III/LAV positive and the remaining, negative specimens. However, a better measure of effective sensitivity than either of these approaches provides would be the ability of assays to identify weakly reactive sera. Individuals who have recently been infected and patients with AIDS sometimes have very low anti-HTLV-III/LAV titres, and their sera should be stored for use in a future assessment of assay sensitivities. This study has not produced evidence that any of the commercial assays are inadequately sensitive, but it is possible that blood donors and others will be found with barely detectable or undetectable anti-HTLV-III/LAV who prove to be infectious.[8] If so, it will be important to identify those assays that most readily detect traces of antibody.

The choice of an anti-HTLV-III/LAV assay may also be influenced by other considerations. For instance, many laboratories will find beads or strips of wells more convenient than 96-well plates, and transfusion laboratories which process blood on the day of donation will prefer the more rapid assays (table IV). Assays that involved repeated, closely timed, or intricate manoeuvres were not liked by our operators and may prove less popular than the more straightforward ones. As well as being extra technical work, every additional manipulation introduces another chance of error. The need to replace kits during the study suggests that some ELISA kits may be prone to unacceptable batch variation, and the product consistency of all kits should be carefully monitored.

The first commercial anti-HTLV-III/LAV assays to be licensed by FDA are methodologically identical (type I, fig 1) and so can not legitimately be used to confirm each other. Two of them appear to be only moderately specific and, used as screening tests, will generate many positive reactions. Simpler alternatives to the western blot procedure are needed to examine these and we suggest the use of a type II assay to confirm positive reactions produced by a type I and vice versa. Discrepant results between these assay types will make further tests necessary—eg, type III assay, indirect immunofluorescence,[5] and western blot.

These evaluations have shown that while some commercial assays need to be refined others, taken together, offer the accuracy and methodological diversity necessary for a satisfactory anti-HTLV-III/LAV testing scheme. Sets of the evaluation specimens will be examined by additional commercial kits as they become available, but at present C, D, and E seem to be the most suitable assay kits for clinical laboratories, mainly because of their superior specificity. Of these, C and E are being used to examine larger numbers of blood donations, to establish whether they will be suitable as routine screening tests in the British National Blood Transfusion Service. Arrangements for confirmatory testing are not yet complete, but in the UK complementary tests by type I and type II assays, supplemented as required by other serological procedures, will probably be used to verify positive and suspect results.

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A fuller analysis of the results of this study may be obtained from Mr D. A. Kennedy, Supply Division, DHSS, 14 Russell Square, London WC1B 5EP (enclose £10 for airmail postage). The study was funded by the Supply Division, Department of Health and Social Security.

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ADDENDUM

Immunofluorescence and Western Blot Assays

The specimens were heat treated and randomised, and then tested under code by Dr M. S. Pereira (Virus Reference Laboratory) for immunofluorescence antibody to HTLV-III.[5] The specimens were also sent, randomised and under code, but unheated to a commercial facility for western blot analysis. The results were reported as reactive/non-reactive against two protein bands of HTLV-III, p24 and gp41. For 2 specimens that were p24 and/or gp41 reactive a reaction against p55 was noted. Specimens reactive against either or both p24 and gp41 were regarded by the company as containing anti-HTLV-III.

Immunofluorescence

All 220 *blood donor* specimens were anti-HTLV-III negative. In the *high risk group* 69 specimens were anti-HTLV-III positive.

Reported as negative were the 11 specimens thought to be anti-HTLV-III negative on the basis of solid phase assay results and 3 others. In the *potential false positive group* 55 specimens were anti-HTLV-III negative and 2 were positive (a rheumatoid-factor-containing serum and a chronic lymphatic leukaemia specimen).

The occurrence of only 3 probable false negative and only 2 probable false positive results out of 360 indicates that, in experienced hands, immunofluorescence antibody testing would be a useful confirmatory procedure if used in conjunction with at least one other confirmatory test for anti-HTLV-III/LAV. However, it is a subjective test and the number of false-negative reactions suggests that it may be too insensitive for screening purposes.

TABLE V—WESTERN BLOT TESTING OF BLOOD DONOR SPECIMENS

Serum	Protein band		
	p24	gp41	p55
Reactive sera (n=5)			
1	+	–	NR
2	+	–	NR
3	+	–	+1
4	–	+	NR
5	+	–	+2
Reactive serum (p55 only)	–	–	+1
Non-reactive sera (n=214)	–	–	NR

NR = not reported.

Western Blot

5 of the 220 *blood donor* specimens were reported to contain anti-HTLV-III. A sixth reacted against p55 (table V). In the *high risk group* 73 specimens were reported to contain anti-HTLV-III, including 72 that were positive by the seven solid phase assays. 67 reacted against both p24 and gp41, 3 against p24 only and 3 against gp41 only. 1 of the p24+, gp41− specimens was anti-HTLV-III negative in all seven solid phase assays. The remaining 10 high risk group specimens, which were also negative by the seven solid phase assays, were non-reactive by western blot. All 57 *potentially false positive* specimens were reported anti-HTLV-III negative.

Thus there were 5 western blot positive blood donor specimens that were negative in all the phase 1 anti-HTLV-III solid phase assays. These 5 and 1 high risk group specimen probably showed false positive western blot reactions. None of these 6 specimens reacted against both p24 and gp41 bands whereas 67 (93%) of the 72 high risk group specimens thought to be truly anti-HTLV-III positive did. There were apparently no false-negative western blot results. The findings suggest that if either the p24 or the gp41 band does not react it is unwise to rely on western blot results for anti-HTLV-III. In this study 6 out of 11 specimens reacting with only one of these two bands gave apparently false-positive results.

Occasional Survey

PROLONGED EXPIRATORY APNOEA AND IMPLICATIONS FOR CONTROL OF BREATHING

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IMPORTANT differences exist between clinical physiological observations made in a wide range of respiratory disorders and the commonly accepted view that breathing is controlled by an intrinsic CNS inspiratory rhythm generator, mainly modulated by chemical factors. Studies of sleep-related disorders have shown that gross arrhythmias in breathing are common. In some patients the ensuing severe asphyxia cannot stimulate or restore effective breathing unless the patient wakes up, even though in the waking state chemical control of breathing is more or less normal by conventional tests.[1] Nonetheless most investigators still seem committed to the view that chemoreception is pre-eminent in respiratory control. How has this come about?

NEGLECT OF VAGAL REFLEX CONTROL

More than 100 years ago Breuer and Hering described a group of reflexes in their treatise on the "self steering of respiration through the nervus vagus".[2] The reflex least often referred to, and yet perhaps the most remarkable, is the active expiratory response which is directly related to the magnitude of lung inflation when the vagi are intact. Not only was its physiological existence questioned at the time (it was presumed to be an artifact of the opioids used for anaesthesia), but also its relevance has not been recognised even though the neuromuscular pathways involved were clearly defined in 1972.[3] The discovery of chemoreceptors by Heymans in 1933[4] has dominated respiratory physiology ever since. As a result the methods normally used for the study of respiratory control have involved, in laboratory animals, deep anaesthesia, elimination of the upper airways by tracheal cannulation, vagotomy, and often thoracotomy (or, in man, mouthpiece breathing), and have left little place for vagal reflex control to be properly considered. Add to this the popular view that apnoea in response to lung inflation is present only for the first few days of life,[5] and the disregard for vagal mechanisms in respiratory control is virtually complete. This is despite the observation that, with vagal blockade, respiratory physiologists have prolonged their breathholding to frank cyanosis.[6] Thus, even after decades of investigation of the fine control of rhythmogenesis there is no satisfactory explanation of disordered breathing rhythm, which is very common in respiratory disease and sleep.

Nowadays investigators try to identify a sleep-related disorder of chemical control even in conditions such as the obstructive sleep apnoea syndrome. It is assumed that respiratory control depends almost exclusively on chemical factors during slow wave sleep (SWS).[7] During rapid-eye-movement (REM) sleep regulation has been considered abnormal[8] or even paradoxical.[9] While the regulation of breathing during REM sleep is certainly different, there is no reason to consider it deficient in normal healthy mammals. A rare condition does exist in which central alveolar hypoventilation (Ondine's curse) occurs predominantly during SWS, causing hypoxaemia, hypercapnia, and sometimes apnoea. In one case alveolar ventilation[10] decreased more than minute ventilation did; this suggests disturbed regulation of intrapulmonary ventilation, in which the vagus has a major role. In most sleep-related disorders obstructive, central, or mixed apnoeas occur, with or without associated hypoventilation, which cause secondary disruption of chemical control. These severe secondary sequelae are promptly relieved by tracheostomy or positive nasal airway pressure, which permit effective ventilation during sleep. In some patients chemosensitivity and/or effective upper airway function also returns. The central apnoea remained in some patients during sleep even when they were breathing through a tracheostome, whereas expiratory nasal pressure caused a reduction in both obstructive and central apnoea.[11] In both these disorders there is a prolonged active expiratory phase, as shown by accurate measurement of expiratory airflow. Although this has been presented as a unifying hypothesis for the pathophysiology of sleep apnoea, its implications for a revision of respiratory control have not been considered.

NEONATAL BREATHING PATTERNS

The developmental aspects are relevant because it was the rapid waning of the inhibitory (vagal) inflationary response in the newborn and the absence of its response in awake adults breathing normally that contributed to the loss of interest in vagal reflexes.

Newborn babies often generate large positive expiratory pressures spontaneously during their first few breaths.[12] They also generate such pressures when their lungs are artificially inflated during resuscitation; this has been called the "rejection response".[13] The expiratory grunt, as air is