D., Saha, K. C. and Chakraborti, D., *Environ. Geochem. Health*, 1995, (in press).
8. Harding, B., in *Arsenic: Industrial, Biomedical, Environmental Perspectives* (eds Lederer, W. H. and Fensterheim, R. J.), Van Nostrand Reinhold, New York, 1983, pp. 203–209.
9. Johnson, J. L. and Rajagopalan, K. V., *Bioinorg. Chem.*, 1978, **8**, 439–444.
10. Tabor, M. Wilson, in International Conference on Arsenic in Groundwater: Cause, Effect and Remedy, Jadavpur University, Calcutta, 1995, p. 38.
11. Biagini, R., *La Semena Med.*, 1972, **141**, 812–817.
12. Kinniburgh, D. G., Gale, I. N., Smedley, P. L., Darling, W. G., West, J. M., Kimblin, R. T., Parker, A., Rae, J. E., Aldours, P. J. and O'shea, M. J., *App. Geochem.*, 1994, **9**, 175–195.
13. Welch, A. H., Lico, M. S. and Hughes, J. L., *Groundwater*, 1988, **26**, 333–347.
14. Tseng, W. P., Chu, H. M., How, S. W, Fong, J. M., Lin, C. S. and Yeh, S., *J. Nat. Cancer Inst.*, 1968, **40**, 453–463.
15. Sancha, Ana Maria, November, 1994, Arsenic contamination in northern Chile, evaluation and control, a case study. Final report. Evaluation of the effect of the consumption of arsenic-containing water on Atacane no communities, Universidad de Chile.
16. Astolfi, E., Maccagno, A., Garcia Fernamdez, J. C, Vaccaro, R. and Stimola, R., *Biol. Trace Element Res.*, 1981, **3**, 133–143.
17. Cebrian, M. E., Albores, A., Aguilar, M. and Blakely, E., *Human Toxicol.*, 1983, **2**, 121–133.
18. Nakorn Si Thammarat Provincial Health Office, Ministry of Public Health, January 1, 1988, Conclusion of control measure action on arsenic poisoning in Ronpiboon district Nakorn Si Thammarat Province, Public Health Department, Ronpiboon, Thailand.
19. Farmer, J. G. and Johnson, L. R., *Bri. J. of Ind. Med.*, 1990, **47**, 342–348.
20. Arnold, H. L., Odam, R. B. and James, W. D., *Disease of the Skin Clinical Dermatology*, W. B. Saunders Company, Philadelphia, USA, 1990, 8th edn, 121–122.
21. Ioamid, N., Bors, G., Popa, I. and Dtsch, Z., *Gesamte Gerichtd. Med.*, 1961, **52**, 90–94.

ACKNOWLEDGEMENT. We thank CSIR, New Delhi and SOES, JU for financial support.

Received 10 April 1996; revised accepted 3 May 1996

Solid-phase cellular lectin assay to study surface lectin binding sites on rhesus monkey spermatozoa

D. Navaneetham, P. Sivashanmugam and M. Rajalakshmi

Department of Reproductive Biology, All India Institute of Medical Sciences, New Delhi 110 029, India

Here we report the binding of lectins (Con A, PNA and WGA) to cauda epididymal and ejaculated rhesus monkey spermatozoa using a solid-phase cellular lectin enzyme immunoassay (CLEIA). For standardization of the assay, optimum dilutions of lectins, enzyme conjugated antibodies, inhibitor saccharides and sperm number for coating to microtitre plate wells were established. Using radioiodinated lectins, radio-lectin assay was also established but correlation analysis showed CLEIA had higher sensitivity compared to radio-lectin assay (CRLA). Using CLEIA, WGA binding to ejaculated spermatozoa showed a significant increase compared to cauda epididymal spermatozoa. Using FITC labelled lectins, Con A, PNA and WGA were localized mainly in the acrosomal region of caudal and ejaculated spermatozoa while the post-acrosomal region did not show any fluorescence. The solid phase lectin immunoassay developed in this study with a relatively small number of sperm is simple to perform, sensitive and specific and can be used to screen antisera and to analyse changes in lectin binding to cell surfaces.

A variety of probes including antibodies, lectins and membrane intercalating agents have been used to understand the complex macromolecular organization of the mammalian sperm surface[1,2]. These include semiquantitative and quantitative studies using unlabelled[3–5] or radiolabelled lectins,[6,7] flow cytometry[8], cytochemistry[9–12] and electron microscopy[13]. These studies indicated that the glycoconjugate moieties on sperm surface are involved in spermatogenesis and in post-testicular maturational changes[14] resulting in spermatozoa acquiring fertilizing capacity. These methods, while giving valuable data, have used fluid phase systems which can cause agglutination of cells giving erroneous results. A method using solid phase methods to assess lectin-binding sites on sperm surface would be easy to carry out in large number of samples, unhampered by lectin-induced agglutination seen in fluid phase systems. Here we describe the development of solid-phase CLEIA and CRLA methods for analysing lectin binding to the spermatozoa. Fluorescence localization of the lectins was also carried out to assess changes, if any, in the site of localization of lectins in prefixed caudal and ejaculated spermatozoa.

Materials and methods

Preparation of sperm suspension and coating of microassay plates

Semen was collected by penile stimulation[15] from five adult male rhesus monkeys (*Macaca mulatta*; 8–10 kg) during November to February. Cauda epididymal spermatozoa were collected by retrograde flushing using medium[16] and checked for the absence of erythrocytes and epithelial cells. Motile spermatozoa were collected by the 'swim-up' procedure, washed in phosphate buffered saline (PBS; 75 mM $Na_2HPO_4 \cdot 2H_2O/KH_2PO_4$, 77 mM NaCl, pH 7.2) and resuspended in PBS to obtain the requisite sperm concentration. For standardization, 100 µl of motile sperm suspension was dispensed at the bottom of microtitre plate wells, fixed with 0.1% glutaraldehyde in PBS for 30 min at 4°C and left at 37°C for 12–16 h for attachment. Microscopic evaluation showed glutaraldehyde fixation did not affect lectin distribution. Washing of microtitre plates, wherein spermatozoa were fixed after coating, gave a cell loss of 0.38% of cells compared to 10.8% loss when fixed spermatozoa in PBS were added to wells. Since cell fixation after coating gave better absorbance values (1.06 ± 0.07; $n = 30$ for Con A) compared to fixation before coating (0.397 ± 0.01), the former method was used subsequently.

Cellular lectin immunoassay

The sperm-coated plates were washed thrice with washing buffer (PBS containing 0.05% Tween 20). Non-specific binding was blocked for 3 h using 400 µl of blocking buffer (PBS containing 1% BSA). The blocking buffer was removed and the cells were incubated at 37°C for 60 min with 100 µl of lectins (2.5–5 µg/ml). Unbound lectins were removed, the wells washed thrice with washing buffer, and 100 µl of appropriately diluted horse radish peroxidase (HRP) coupled anti-lectin antibody was added. The plates were incubated following the addition of 100 µl of substrate solution (34.7 mM citric acid, 75 mM $Na_2HPO_4 \cdot 2H_2O$, 0.04% *o*-phenylenediamine and 0.03% H_2O_2, pH 5.0). The assay was terminated by adding 50 µl of 2.5 N sulphuric acid. The absorbance was measured at 490 nm (Emax Precision Microplate Reader, Molecular Devices, USA).

Cellular radiolectin assay

Concanavalin A (Con A), peanut agglutinin (PNA) and wheat germ agglutinin (WGA) were radioiodinated using chloramine T (ref. 17). ^{125}I-labelled lectins (5×10^4 cpm; 0.15–0.25 µg) and 100 µM of sodium iodide in blocking buffer were added to sperm-coated wells (in duplicate), after blocking nonspecific binding, and incubated at 37°C for 60 min. Unbound lectins were removed, the wells washed repeatedly, incised and counted in a Gamma counter.

Inhibitor ligand and displacement study

The specificity of lectin binding was assessed ($n = 4$, in duplicate) by incubating (37°C, 30 min) with different concentrations (up to 300 mM) of appropriate inhibitor saccharides, prior to their addition to the wells. In subsequent studies, the inhibitor sugar that gave maximum inhibition (α-methyl mannose for Con A and galactosamine for PNA) was used to identify the best procedure for inhibition studies, by the following three methods of incubation (37°C): (i) Lectin, preincubated with the inhibitor was added to the wells and incubated for 30 min; (ii) Lectin and inhibitor were added to wells simultaneously and incubated for 30 min; (iii) The coated plates were incubated with lectin for 15 min, the inhibitor was added and incubated for 15 min.

Localization of lectins

Sperm suspension (1 million) in PBS was fixed (15 min) by the dropwise addition of 4% paraformaldehyde and processed for lectin localization[12]. The fluorescence was visualized under an epifluorescence microscope (Laborlux S. Wild Leitz GmbH, Germany) using ploemopack I2 filter block (excitation filter BP 450–490 and suppression filter LP 515).

Statistical analyses

Data were analysed for correlation coefficient and significance of correlation by Pearson's correlation method and one-way ANOVA.

Results

Ejaculated spermatozoa collected by 'swim-up' procedure showed rapid, linear progressive motility and the absence of morphological abnormalities in Papanicolau stained smears. Coated microplates left at 37°C for 12–16 h showed firm attachment of cells ($n = 3$).

Standardization of concentration of lectin and HRP conjugated anti-lectin antibodies

Experiments ($n = 3$, in duplicate) to determine the op-

timum concentration of lectins and the respective HRP-coupled anti-lectin antibodies using sperm concentration of 0.1×10^5/well (for Con A and PNA) and 0.5×10^5/well (for WGA) gave the following data: (i) Using different dilutions of Con A (1 : 100–1 : 12,800) and HRP-anti-Con A antibody (1 : 100–1 : 25,600), optimum results were obtained using Con A up to a dilution of 1 : 800 and HRP-anti-Con A antibody up to a dilution of 1 : 400 or 1 : 800 (Figure 1 *a*); (ii) Using six dilutions of PNA (1 : 100 to 1 : 3,200) and five dilutions of HRP-anti-PNA antibody (1 : 100 to 1 : 1,600), decrease in absorbance values (Figure 1 *b*) was seen with increasing dilutions, but the results were not markedly different when PNA and its antibody were used at dilutions of 1 : 100 to 1 : 400; (iii) Similarly, WGA and anti-WGA antibody at dilutions from 1 : 100 to 1 : 400 gave similar results (Figure 1 *c*) and both were used at 1 : 200 dilutions, for further studies.

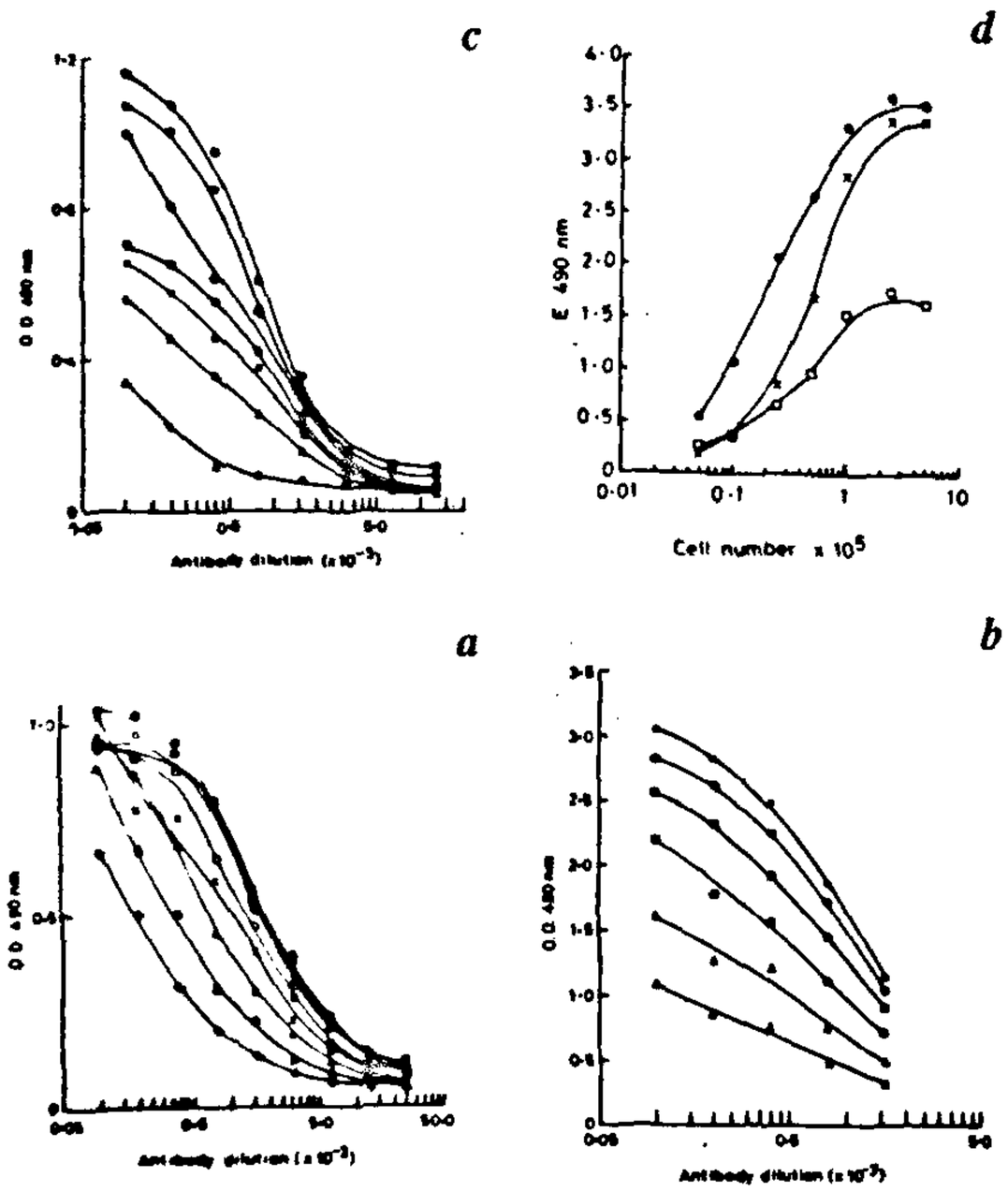


Figure 1 *a–d*. *a*, Determination of the optimum concentration of Con A and HRP-anti-Con A using 0.1 × 105 ejaculated sperm per well. Con A dilutions are: ●—● 1 : 100, O—O 1 : 200, ■—■ 1 : 400, □—□ 1 : 800, × —× 1 : 1,600, Δ—Δ 1 : 3,200, s—s 1 : 6,400, + — + 1 : 12,800. *b*, Determination of the optimum concentration of PNA and HRP-anti-PNA Ab using 0.1×10^5 ejaculated sperm per well. PNA dilutions are: ●—● 1 : 100, O—O 1 : 200, ■—■ 1 : 400, □—□ 1 : 800, ▲—▲ 1 : 1,600, Δ—Δ 1 : 3,200. *c*, Determination of the optimum concentration of WGA and HRP-anti-WGA Ab using 0.5×10^5 ejaculated sperm per well. PNA dilutions are: ■—■ 1 : 100, O—O 1 : 200, ●—● 1 : 400, □—□ 1 : 800, × —× 1 : 1,600, Δ—Δ 1 : 3,200, ▲—▲ 1 : 6,400. *d*, Increase in optical density and plateauing when increasing cell numbers ($0.05–5.0 \times 10^5$) were exposed to Con A ●—●, PNA × —× and WGA □—□ and their respective HRP anti-lectin antibodies.

Standardization of cell number

When increasing number of coated cells ($0.05–5 \times 10^5$) were exposed to Con A, PNA and WGA and to their respective HRP-anti-lectin antibodies (1 : 400 for Con A and PNA and 1 : 200 for WGA), even at a low cell number of 0.05×10^5, detectable absorbance was visible indicating the sensitivity limit of the assay (Figure 1 *d*); increase in cell number resulted in increase in absorbance, until lectin concentration became limiting.

Specificity of the cellular lectin immuno assay

α-Methyl mannose was a more efficient inhibitor for Con A than D-mannose since optimum inhibition was obtained between 50 and 100 mM of α-methyl mannose whereas D-mannose up to a concentration of 200 mM did not give complete inhibition. PNA binding was completely inhibited by 50 mM galactosamine. Complete inhibition of PNA and WGA binding was not achieved by 300 mM of *N*-acetyl galactosamine and *N*-acetyl glucosamine, respectively. Preincubation of Con A with α-methyl mannose or PNA with galactosamine gave better inhibition than simultaneous addition of lectin and inhibitor or addition of inhibitor after exposing the sperm to lectin but, at higher concentrations of galactosamine, the results of the three methods were similar.

Radiolectin assay

The specific activity of labelled lectins was 3.2×10^5 cpm/μg for Con A, 2.3×10^5 cpm/μg for PNA and 2.02×10^5 cpm/μg for WGA. A gradual increase in labelling occurred when cell concentration was increased and the radioactivity-bound plateaued at 1×10^5 sperm/well, for the three lectins; further increase in cell number up to 10×10^5 did not increase lectin binding.

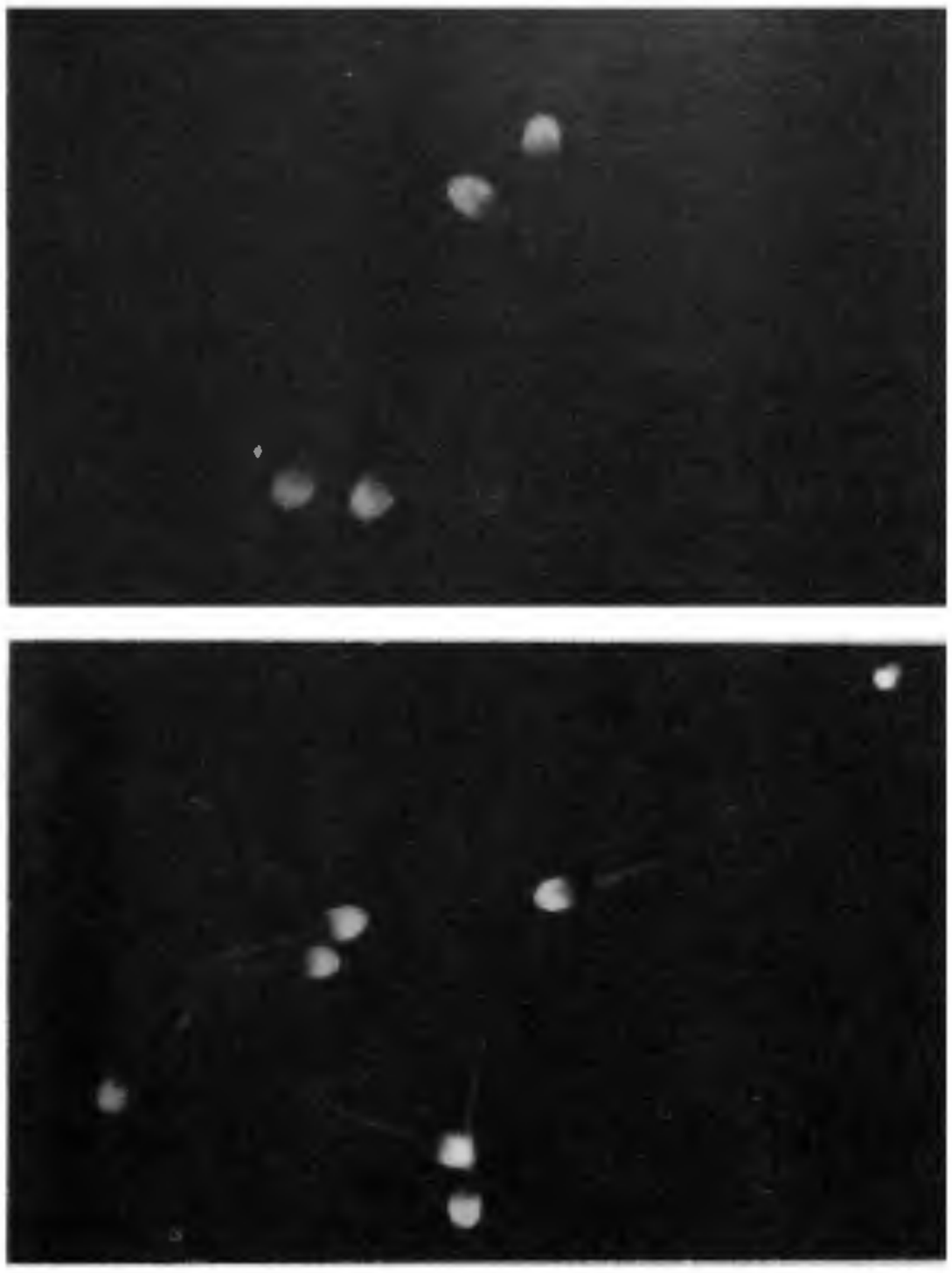
Binding of lectins to rhesus monkey spermatozoa

Of the three lectins used, binding (expressed as optical density; mean ± SE) of Con A to caudal spermatozoa (1.47 ± 0.02; $n = 56$) was maximum followed by WGA (0.63 ± 0.01; $n = 38$) and PNA (0.58 ± 0.02; $n = 42$) binding. Spermatozoa at 1×10^4/well were used for Con A and PNA binding and at 5×10^4/well for WGA binding. A significant ($p < 0.05$) increase in WGA binding (1.41 ± 0.04; $n = 42$) was seen in ejaculated spermatozoa compared to caudal sperm; but the increase in Con A (1.79 ± 0.08; $n = 42$) or PNA (0.61 ± 0.01; $n = 40$) binding to ejaculated spermatozoa, compared to caudal sperm was not significant. Correlation coefficient and significance of correlation between solid-phase CLEIA and

CRLA were $r = 0.84$ (Con A; $p < 0.001$); $r = 0.85$ (PNA; $p < 0.001$) and $r = 0.92$ (WGA; $p < 0.001$).

Lectin localization

Paraformaldehyde fixed cauda epididymal (Figure 2) and ejaculated (Figure 3) spermatozoa showed localization of FITC–Con A in the acrosomal region while the equatorial region and midpiece showed light lectin localization; the postacrosomal region, neck and tail did not show any fluorescence. Similar pattern of lectin localization was seen with FITC–PNA and FITC–WGA.



Figures 2 and 3. Intense localization of FITC-Con A in the acrosome and light fluorescence in the midpiece of cauda epididymal and ejaculated spermatozoa respectively (× 1000).

Discussion

Here we describe a simple, specific and sensitive solid phase cellular lectin assay which was used to study binding of lectins to cauda epididymal and ejaculated spermatozoa, using a small number of cells. This assay has a higher sensitivity limit since detectable results were obtained using low sperm concentration (0.05×10^5/well) whereas earlier studies have used a much higher concentration of cells[3,7,8]. Other investigators have carried out semiquantitative and quantitative measurement of lectin-binding by agglutination of sperm cells[5], by assessing binding capacity of radiolabelled succinylated Con A to sperm surface[7] and by flow cytometry[8]. Quantitation of changes in lectin binding by the earlier methods has certain disadvantages which include nonavailability of all surface-lectin-binding sites due to agglutination and the expensive equipment needed for flow cytometry. Susko-Parrish *et al.*[7] attributed agglutination of sperm cells as one of the complicating factors for their inability to obtain saturable binding kinetics. The method of using sperm-coated microtitre plates developed in this study has ensured the availability of a relatively larger proportion of sperm surface lectin-binding sites, which may explain the saturability of binding, as observed by the plateauing of absorbance values with increased concentration of lectin or spermatozoa. The method of obtaining sperm-coated plates by evaporation can be done in large number of samples and is easier since centrifugation is not necessary while other methods[18,19] require centrifugation to maximize cell binding to the microwell plates. Statistical evaluation of data obtained using the solid-phase method either as a CLEIA or as a CRLA shows that the sperm-coated plates can be used for both assays with comparable results. Since CLEIA does not require the use of isotopes and the assay is easy to handle it may be a more useful technique for routine purposes in contrast to solid-phase CRLA. Using the solid-phase CLEIA, the present study has shown that increase in WGA binding occurs during ejaculation of spermatozoa, perhaps due to interactions with accessory gland secretions. The pattern of localization of FITC labelled lectins mainly in the acrosomal region of sperm, is in conformity with the published literature. CLEIA has been used by us as a more sensitive system than conventional Ouchterlony[20] to screen polyclonal antisperm antisera with good results.

1. Holt, W. V., *Int. Rev. Cytol.*, 1984, **87**, 159–194.
2. Eddy, E. M., in *The Physiology of Reproduction* (eds Knobil, E. and Neill, J. D.), Raven Press, New York, 1988, vol. 1, pp. 27–68.
3. Hammerstedt, R. H., Hay, S. R. and Amann, R. P., *Biol. Reprod.*, 1982, **27**, 745–754.
4. Nicolson, G., Lacorbiere, M. and Yanagimachi, R., *Proc. Soc. Exp. Biol. Med.*, 1977, **141**, 661–663.
5. Olson, G. E. and Danzo, J., *Biol. Reprod.*, 1981, **24**, 431–443.
6. Edelman, G. M. and Millette, C. F., *Proc. Natl. Acad. Sci. USA*, 1971, **68**, 2436–2440.
7. Susko-Parrish, J. S., Hammerstedt, R. H. and Senger, P. L., *Biol. Reprod.*, 1985, **32**, 129–136.
8. Magargee, S., Kunze, E. and Hammerstedt, R. H., *Biol. Reprod.*, 1988, **38**, 667–685.

9. Fenderson, B. A., O'Brien, D. A., Millette, C. A. and Eddy, L. M., *Dev. Biol.*, 1984, **103**, 117–128.
10. Lee, M. C. and Damjanov, I., *Anat. Record.*, 1985, **212**, 282–287.
11. Millette, C. F., in *Immunobiology of Gametes* (eds Edidin, M. and Johnson, M. H.), Cambridge University Press, 1977, pp. 51–71.
12. Schwarz, M. A. and Koehler, J. K., *Biol. Reprod.*, 1979, **21**, 1295–1307.
13. Courtens, J. L. and Fournier-Delpech, S., *J. Ultrastruct. Res.*, 1979, **68**, 136–148.
14. Koehler, J. K., *Arch. Androl.*, 1981, **6**, 197–217.
15. Mastroianni, L. and Manson, W. A., *Proc. Soc. Exp. Biol. Med.*, 1963, **112**, 1025–1027.
16. Biggers, J. D., Whitten, W. K. and Whittingham, D. G., in *Methods in Mammalian Embryology* (ed. Daniel, J. C.), Academic Press, New York, 1971, pp. 86–116.
17. Greenwood, F. C., Hunter, W. M. and Gliver, J. S., *Biochem. J.*, 1963, **89**, 114–123.
18. McArthur, C. P., Sengupta, S. and Smith, R. T., *J. Immunol. Methods*, 1981, **43**, 343–348.
19. Paul, S., Baukloh, V. and Mettler, L., *J. Immunol. Methods*, 1983, **56**, 193–199.
20. Ouchterlony, O., in *Progress in Allergy* (eds Kallos, P. and Waksman, B. H.), Karger, Basel, 1962, vols. VI, II, pp. 30–145.

ACKNOWLEDGEMENTS. This work was supported by grants from the Indian Council of Medical Research and Council of Scientific and Industrial Research, Government of India. D.N. and P.S. thank University Grants Commission, New Delhi and AIIMS, respectively, for the award of Junior Research Fellowships. M.R. thanks World Health Organization, Geneva for training in advanced sperm function tests.

Received 6 February 1996; revised accepted 27 March 1996

^{40}Ar–^{39}Ar ages of Anjar Traps, Western Deccan Province (India) and its relation to the Cretaceous–Tertiary Boundary events

T. R. Venkatesan, Kanchan Pande and Z. G. Ghevariya*

Physical Research Laboratory, Ahmedabad 380 009, India
*Geological Survey of India, Gandhinagar 382 042, India

^{40}Ar–^{39}Ar ages of whole rock basalt samples from the lava flows sandwiching the Ir-rich intertrappean at Anjar are indistinguishable from the Cretaceous/Tertiary Boundary (KTB) age. These results imply that the Ir-rich layer represents the KTB boundary layer. The presence of several flows below the lower dated flow supports our earlier observation[1], based on the geochronological studies of Western Ghat lava flow sequence, that the initiation of the Deccan volcanism predated KTB.

THE discovery of an Ir-rich layer in an intertrappean bed at Anjar, Western India[2], seeks to establish a relationship, if there is, between the KTB events and the Deccan volcanism. The Deccan volcanism is cited as the internal cause for the KTB events[3–5], although there is mounting evidence that the iridium anomaly in the KTB layer is due to an impact of a bolide on earth[6]. A majority of physico-chemical evidences available today (such as shocked quartz, meteoritic spinels, geochemical anomalies and identification of the Chicxulub crater as the probable impact site) favour the impact scenario for the KTB event[7–9]. A third view is that the impact and volcanic events at the KTB are independent that occurred by chance at the same time[10]. Thus, the debate continues as to the cause of the KTB transition being internal or external. Therefore, it is important to get some high resolution event sequence from intertrappeans of the Deccan Province.

The geochemical observations on both the Deccan Traps (DT) and intertrappean sequences considered with the $^{40}Ar/^{39}Ar$ ages on the DT rule out a link between the KTB and DT[1,11,12]. The interpretation of the geochronological data, however, is not conclusive. For example, some workers[13–17] suggest that the available 'good' quality ^{40}Ar–^{39}Ar ages coupled with palaeomagnetic data imply a rapid eruption of Deccan lavas coincident with the KTB. On the other hand, Venkatesan *et al.*[1] argue that the initial, most intense, pulse of Deccan eruption predated the KTB by at least 1 Ma and the total duration of the volcanism is not less than 3 Ma. The conflicting interpretations of a similar data set stems primarily from the uncertainties in the geomagnetic time scales (GMTS) and the age(s) of the monitor sample(s) used for the ^{49}Ar–^{39}Ar dating of the KTB and the DT. These problems could be resolved if the KTB layer within the Deccan province be identified and the ages of the lava flows with respect to the KTB be obtained. Though the KTB