Qualitative identification of type II antibody function in rabbit antihuman IF–B$_{12}$ complex antiserum and its cross reactivity

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The presence of the binding (type II) antibody function in rabbit antisera raised against purified human intrinsic factor–vitamin B$_{12}$ complex (IF–B$_{12}$ complex) has been qualitatively identified by an *in vitro* assay employing zirconyl phosphate gel. A sample of human autoimmune antisera to human IF did not exhibit the type II antibody function by the same assay. Cross-reactivity studies employing either the rabbit or the human antisera did not show immunological sequence interrelationship between human, rat and rabbit intrinsic factor–vitamin B$_{12}$ complexes or with hog IF. A possible link between the binding epitope (type II epitope) on the IF molecule and immunological cross reactivity is suggested.

SCHWARTZ[1], Garrido-Pinsen *et al.*[2] and Taylor[3] suggested that serum from patients with pernicious anemia contains antibodies against intrinsic factor (IF). Lowenstein *et al.*[4] confirmed the presence of IF inhibiting substances in the serum of patients with orally-treated pernicious anemia. Jefferies *et al.*[5] demonstrated that an autoimmune antibody that destroys the intrinsic factor activity of human gastric juice was present in about 30% of patients with pernicious anemia. These autoimmune antibodies are functionally of two kinds, both of which have been reported to be present in the IgG fraction of the serum. The type I or 'blocking antibody' prevents vitamin B$_{12}$ from complexing with intrinsic factor, and type II or 'binding antibody' prevents the attachment of intrinsic factor to the receptor sites in the ileum[6,7]. These autoimmune antibodies are rarely found in conditions other than pernicious anemia, making them physiologically more specific[8].

An assay for the rapid estimation of blocking antibodies (type I), employing zirconyl phosphate gel (z-gel) had been developed and described by Hansen *et al.*[9]. The gel generally adsorbed proteins at or below their isoelectric point, but quantitatively adsorbed the intrinsic factor activity of human gastric juice at pH 5. At a pH greater than 5.5, the IF–B$_{12}$ complex does not get adsorbed on to the gel as the negative charge on the molecule becomes greater. Nevertheless, in the presence of an antibody binding the IF–B$_{12}$ complex, a greater net positive charge makes possible the adsorption of the 'binding antibody IF–B$_{12}$ complex' onto the gel at pH 6.25. This method, therefore, provided a sensitive *in vitro* assay[10] for the estimation of IF–B$_{12}$ complexes from different species.

Using the z-gel assay method, the presence of the 'binding type antibody' (type II autoimmune antibody) in rabbit antisera raised against purified human IF–B$_{12}$ complex was assessed. By the same method human autoimmune antisera to human intrinsic factor, was also assessed for the presence of the type II autoimmune antibody function. In employing these methods, inhibition of vitamin B$_{12}$ binding ability of human gastric juice, and rat gastric IF preparations was noted, that suggested the presence of 'blocking antibody', type I function in both these antisera.

The studies reported here suggest that the binding antibody combining region of IF is essential for the 'antibody–IF–B$_{12}$ complex' formation. Any alterations to this combining region can lead to a loss in ability to form antigen–antibody immunoprecipitation complexes; and perhaps to a loss of the ileal receptor binding ability of the IF–B$_{12}$ complexes.

Agar–agar and Freund's complete adjuvant were obtained from Difco Laboratories, USA. Human serum autoimmune antibody was supplied by V. I. Mathan, Dept of Gastroenterology, Christian Medical College and Hospital, Vellore. All other chemicals used were of analytical grade. Radioactive [^{57}Co] cyanocobalamin (282 µCi/µg) was obtained from Bhabha Atomic Research Centre, India. Pepsin, trypsin, a-chymotrypsin, carboxypeptidase-A, leucine aminopeptidase and hog IF concentrate were obtained from Sigma, USA.

For the preparation of rabbit antisera to human IF–B$_{12}$ complex, an adult normal healthy, male rabbit that weighed about 1 kg was used to raise antibodies against purified human IF–vitamin B$_{12}$ complex. Human IF that was purified 1035-fold by affinity chromatography on vitamin B$_{12}$-sepharose[11,12], and having a specific vitamin B$_{12}$ binding ability of 24 µg B$_{12}$/mg protein was used for the immunization process. Human IF–B$_{12}$ complex (containing 350 µg protein saturated with 8.05 µg B$_{12}$) was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into the dorsal skin of the rabbit in several spots, injecting 0.25 ml of the mixture per spot. The rabbit was maintained on a normal diet thereafter for 15 days. On the 16th day, a single booster dose of IF–B$_{12}$ complex (protein complexed with 0.26 µg B$_{12}$) in a total volume of 1.0 ml, containing 0.5 ml of Freund's complete adjuvant was injected into the foot pads of the rabbit. Immunized rabbit blood (2.0 ml) was collected 3 days later from the ear lobes of the rabbit, and the antiserum was separated by allowing the blood to clot at room temperature. The serum sample was then centrifuged at 3,000 rpm in an MSE table-top centrifuge for 10 min, and the clear serum was decanted, portioned and stored at –20°C for further use.

For protease treatment of human IF-B_{12} complex, affinity-purified human IF-B_{12} complex (24 µg B_{12}/mg protein)[11,12] was treated sequentially with pepsin, trypsin, a-chymotrypsin, carboxypeptidase-A and leucine aminopeptidase, at their optimum pH and at 37°C; maintaining an enzyme to protein ratio of 1:100. Protein samples were adjusted to pH 1-2 with 1 N HCl followed by the addition of pepsin and incubation for 2 h. The enzymes, trypsin through leucine aminopeptidase, were then added after adjusting the pH of the reaction mixture to 7-8 with 1 M NaOH. The incubation was allowed thereafter for a further 4 h. At the end of the incubation, samples were taken for immunodiffusion experiments.

The immunodiffusion experiments were carried out on agar gel plates by the method of Ouchterlony[13]. Solutions of agar–agar (1.5% and 2%) were prepared in 0.85% sodium chloride, and agar gel plates were made in small petri dishes. The antibody serum was placed in the central well and the various antigen samples were applied to the surrounding wells. The plates were developed at 6°C for 48 h.

Inhibition studies of vitamin B_{12} binding by human gastric juice and rat gastric IF preparations were carried out employing the anti IF-B_{12} complex rabbit antiserum, and using the charcoal adsorption method of Gottlieb et al.[14]. Samples (12.4 mg/ml) to be assayed (1:5 diluted) were taken in a total volume of 0.5 ml containing 200 µmoles sodium phosphate buffer, pH 7.5 and varying amounts of 1:20 diluted rabbit antiserum. The reaction mixture was incubated at 37°C for 15 min. At the end of the incubation period, 3.5 ng [57Co]–vitamin B_{12} (specific activity:0.143 µCi/µg) was added to each reaction tube and the tubes were incubated for an additional 15 min. A suspension (2.0 ml) of protein-coated charcoal was then added to each tube to adsorb the free vitamin B_{12}. The tubes were then centrifuged at 1000 g for 15 min, the supernatant was decanted and taken for measurement of radioactivity in a Packard gamma counter.

The effect of human anti-intrinsic factor autoimmune antibody serum on vitamin B_{12} binding by human gastric juice and by rat IF preparation was assessed by the same procedure as described for using the rabbit antiserum.

Zirconium gel (z-gel) was prepared according to the method of Hansen et al.[9]. Zirconium oxychloride (20 g) was taken in 3 l of 0.1 N HCl, and 40 ml of orthophosphoric acid was added to precipitate zirconyl phosphate. After standing for 5 h at room temperature, the supernatant was decanted and the precipitate was washed with 3 l of glass distilled water to remove excess phosphoric acid. The procedure was repeated five times and the final precipitate was taken in 0.1 M acetic acid. One part of the gel was adjusted to pH 5.0 using concentrated ammonium hydroxide and the other part to pH 6.25, and stored in the cold until further use. A 40% (v/v) preparation of the z-gel was used for all the assays.

The ability of z-gel to adsorb [57Co]–vitamin B_{12} complexed to intrinsic factor in human gastric juice, and in rat and rabbit IF preparations was initially determined at pH 5.0. A typical reaction mixture contained 200 µmoles of Tris-HCl buffer, pH 7.5; in a total volume of 0.6 ml and varying amounts of the protein to be assayed, to which saturating amounts of [57Co]–vitamin B_{12} in 0.1 µl (specific radioactivity: 0.01 nCi/ng B_{12}) has been added. The reaction mixture was incubated at 37°C; 15 min for human gastric IF, 5 min for rat IF, and 10 s for rabbit IF preparations. At the end of the incubation period, 1 ng nonradioactive vitamin B_{12} was added to the reaction and mixed well to displace loosely-bound radioactive vitamin B_{12} from protein in the sample. A 40% (v/v) suspension of z-gel (12 ml) was then added to each tube, the tubes were capped with parafilm and inverted twelve times for uniform mixing and then centrifuged at 700 g in a MSE tabletop centrifuge for 10 min. The supernatant was discarded and the gel pellet was washed by resuspension in 0.1 M ammonium acetate buffer, pH 5.0, followed by centrifugation. The procedure was repeated twice and the radioactivity
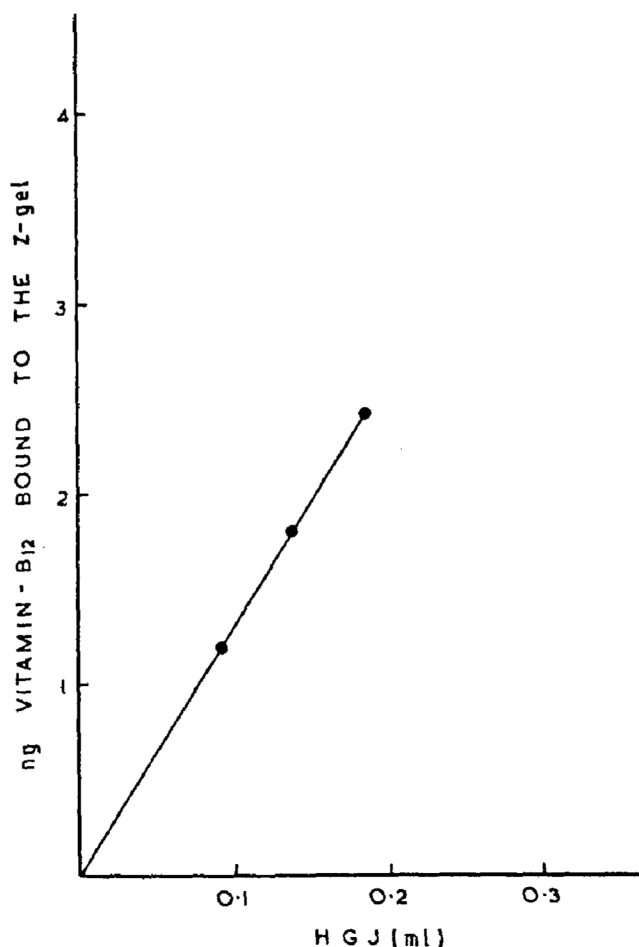

Figure 1. Adsorption of human intrinsic factor–vitamin B_{12} complex by zirconyl phosphate gel at pH 5.0.
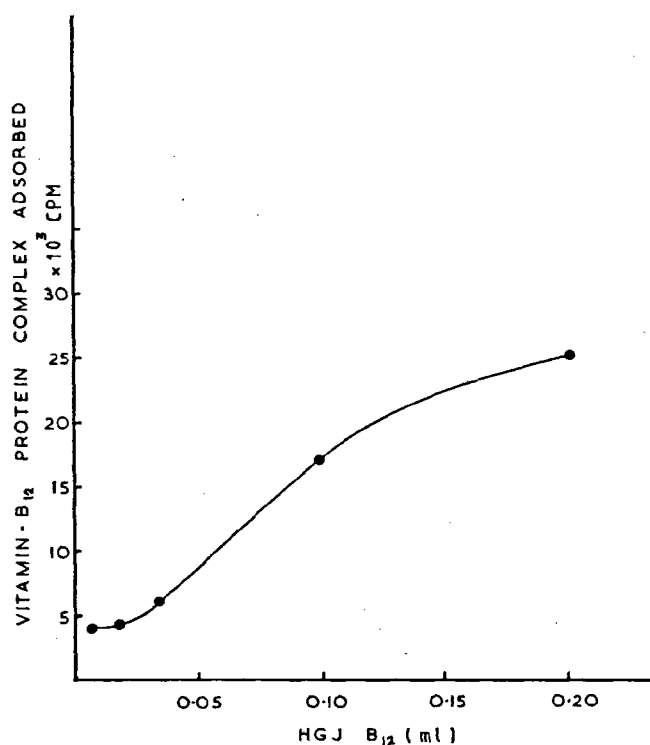
Figure 2. Adsorption of human intrinsic factor–vitamin B_{12} complex by zirconyl phosphate gel at pH 6.25, in the presence of rabbit antihuman IF–B_{12} antiserum.
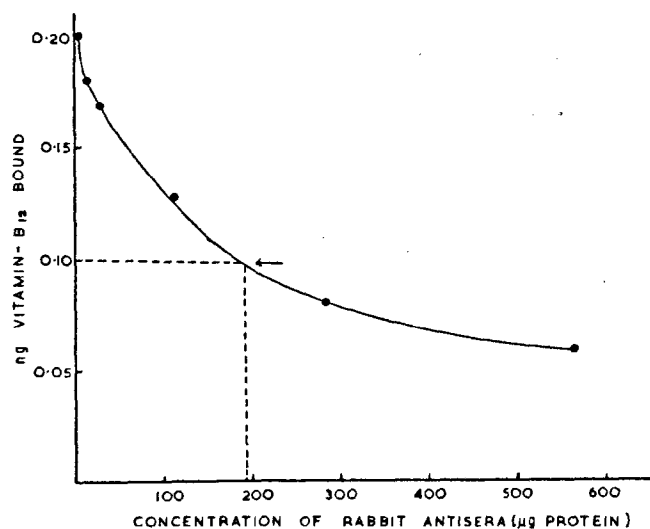

Figure 3. Inhibition of vitamin B_{12} binding to depepsinized human gastric juice as a function of rabbit antihuman IF–B_{12} serum concentration.

adsorbed on the gel was measured in a Packard gamma counter (Figure 1).

The assay using z-gel at pH 6.25 was similar to that described for the z-gel assay at pH 5.0. The experiments

were aimed at quantitating human IF–B_{12} complex as its antibody complex employing the rabbit and human antisera.

For the adsorption of rabbit antibody–human IF–B_{12} complex, varying volumes from a sample of human gastric juice diluted 1:10 and having a specific activity 0.013 µg B_{12}/mg protein, was taken in a total reaction volume of 0.5 ml containing 150 µmoles sodium chloride. Radioactive [^{57}Co]–vitamin B_{12} (0.148 nCi/ng) was added (1.4 ng in 20 µl) to the reaction volume and incubated for 15 min at 37°C. Rabbit antiserum (40 µl of 1:20 diluted solution containing 0.190 mg protein) that gives a 50 per cent inhibition of vitamin B_{12} binding by human gastric juice was added to the reaction volume in each tube. The tubes were incubated for a further period of 15 min. At the end of the incubation, 10 ml suspension (40% v/v) of z-gel at pH 6.25 was added to each tube, the contents of the tubes thoroughly mixed by inverting several times and the tubes were centrifuged at 700 g for 15 min. The supernatant obtained was discarded and the gel pellets were washed in 0.1M ammonium acetate, pH 6.25, three times. The radioactivity adsorbed onto the gel in each tube was measured using a gamma counter. A plot of radioactivity adsorbed on the gel vs concentration of gastric IF-bound B_{12} taken was constructed, from which the amount of B_{12} bound by the gel was estimated. Suitable controls containing the antisera alone were found to adsorb 15% of the initial total radioactivity of the reaction mixture (Figure 2).

For the adsorption of human antibody–human IF–B_{12} complex, diluted (1:10) antihuman intrinsic factor autoimmune antibody serum (1 mg/ml) was used. A known amount of the antiserum (6 µg protein) giving a 50 per cent inhibition of vitamin B_{12} binding by a 1:10 diluted sample of human gastric juice (specific activity: 0.013 µg vitamin B_{12}/mg protein) was used in the assay, the protocol of which was identical to that of the previous experiment. The amount of radioactivity bound to the z-gel at pH 6.25 was measured in a gamma counter. Suitable controls containing the human antisera were found to adsorb less than 10% of initial total radioactivity of the reaction mixture.

Polyclonal rabbit antihuman intrinsic factor–vitamin B_{12} complex antisera brought about 50% inhibition of vitamin B_{12} binding by human gastric juice (Figure 3) and 10% inhibition of vitamin B_{12} binding by rat IF preparations. Human autoimmune antibodies to intrinsic factor also demonstrated a 50% inhibition of vitamin B_{12} binding by human gastric juice (Figure 4), but the inhibition required only 6 µg autoimmune serum proteins as compared to 190 µg rabbit antisera protein. The protein content of human gastric juice sample employed for these studies was 2.15 mg/ml and that of rat and rabbit IF preparations were 11.4 mg/ml and 13.34 mg/ml respectively. The z-gel assay at pH 5.0 demonstrated that
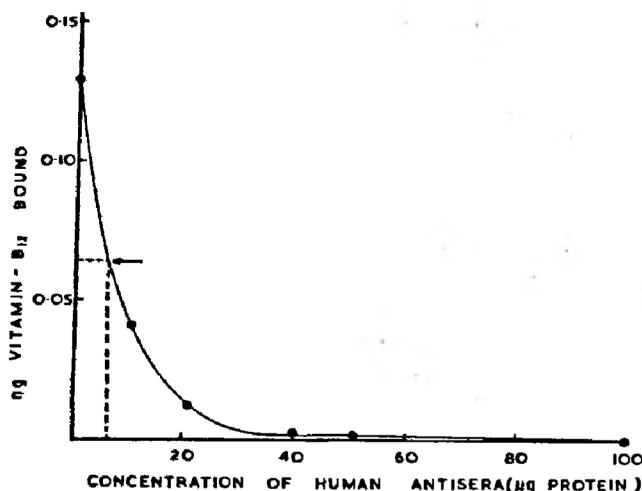
Figure 4. Inhibition of vitamin B_{12} binding to depepsinized human gastric juice as a function of human autoimmune antibody serum concentration.
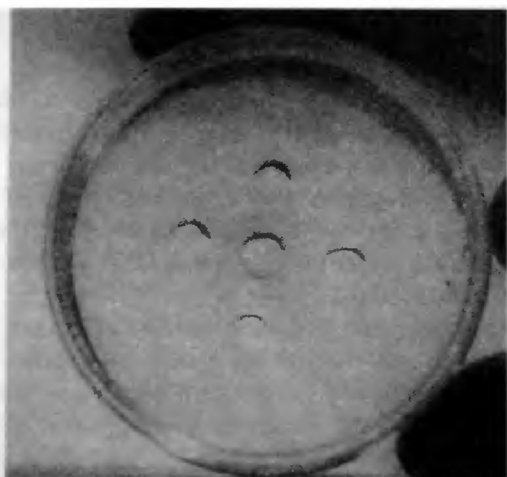

Figure 5. Immunodiffusion using polyclonal rabbit antihuman IF–B_{12} antibody serum (central well contains 4.4 mg antiserum protein in 40 µl). Affinity purified human IF–B_{12} complex was taken (10–50 µl) in the surrounding wells.

human IF from human gastric juice, complexed to vitamin B_{12} was adsorbable on to the gel at that pH (Figure 1). The z-gel assay at pH 6.25 demonstrated that rabbit antisera to human IF–B_{12} complex aided the adsorption of human IF–B_{12} complex by the z-gel at that pH (Figure 2). However, human autoimmune serum was not seen to aid the adsorption of human IF–B_{12} complex by z-gel at pH 6.25.

Polyclonal rabbit antibodies to purified human IF–B_{12} complex immunoprecipitated the complex (Figure 5). Precipitated antigen–antibody complexes on agar under similar conditions were, however, not observed when the rabbit antiserum was cross-reacted with purified IF–B_{12} complexes[12] of rat and

Table 1. Immunodiffusion studies using rabbit antiserum

Sample	Volume range (µl)	Protein range (µg)	Rabbit antisera total protein (mg)	Result
Human gastric juice	50	3.0	3.4	Positive
Purified human IF–B_{12}	10–50	2.65–13.25	4.4	Positive
Proteolysed human IF–B_{12}	10–50	2.0–10.0	4.4	Negative
Purified rat IF–B_{12} (1729-fold)	60–120	4.56–9.12	4.4	Negative
Purified rabbit IF–B_{12} (116-fold)	10–50	5–25	3.9	Negative
Hog IF concentrate	40	9.2	3.3	Negative

rabbit. The rabbit antisera did not immunoprecipitate protease treated human IF–B_{12} complex. Neither did human autoimmune antiserum exhibit immuno precipitation either with human IF or with human IF–B_{12} complex during agar gel immunodiffusion experiments (Table 1).

There is evidence in literature for the existence of two antiintrinsic factor autoimmune antibody types in the serum of patients diagnosed to have pernicious anemia[15]. Of these, the type I, or 'blocking antibody' is reportedly present in 60% of adult cases and the type II, or 'binding antibody', is suggested to occur only in 30% of the cases. It follows, therefore, that the type I antigenic domain on the IF molecule can elicit an antibody response in a host, independent of the type II antigenic domain.

The sample of human autoimmune antibody serum that was capable of inhibiting vitamin B_{12} binding by human gastric juice, but not capable of aiding the adsorption of human gastric juice-bound vitamin B_{12} (HGJ–B_{12}) complex by the z-gel at pH 6.25, shall therefore contain the type I, or the blocking antibody. As the z-gel assay method at pH 6.25 provides a sensitive, in vitro assay, for the IF–B_{12} complex[10], the observation that the rabbit antisera actually aided the adsorption of HGJ–B_{12} complex by the z-gel at pH 6.25, demonstrates the existence of the type II, or the binding antibody function in the rabbit antiserum. Although both human autoimmune antiserum as well as the rabbit polyclonal antiserum were capable of inhibiting vitamin B_{12} binding by human gastric juice, the amount of human auto-immune antibody serum protein that was required to block 50% vitamin B_{12} binding by HGJ was nearly 40-fold less than that required of the rabbit antiserum.

Immunodiffusion studies using the rabbit antiserum did not demonstrate cross reactivity of intrinsic factor from human, rat, rabbit or hog. The antibody binding region (type II region) of IF from these species therefore may not share immunological sequence interrelationship in their antibody binding epitopes. Purified human IF,

390

human IF–B_{12} complex, and a sample of depepsinized human gastric juice, all exhibited a positive immuno-precipitation reaction with the polyclonal rabbit antihuman IF–vitamin B_{12} complex antiserum. Proteolysed human IF–B_{12} complex on the other hand, yielded a negative result when subjected to immunodiffusion. Probably, the antigenic domains on the IF molecule responsible for immunocomplexation underwent structural alterations as a consequence of proteolysis of the IF–B_{12} complex, with concomitant loss of the antibody binding function.

These studies suggest that the type II binding antibody recognition site on the IF molecule may have a role to play in the binding and immunoprecipitation of free IF or IF–B_{12} complex from human source. Either structurally different or the lack of a type II region in the rat, rabbit, and hog IF probably renders these molecular species immuno inactive. Dissimilar type II epitopes of IF from different species may therefore be responsible for the species specificities observed when employing IF for clinical use. Maintaining the structural integrity in the type II domain of IF may also be crucial in the recognition and binding of IF–B_{12} complexes by the IF specific ileal receptors.

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Antiquity of the Narmada *Homo erectus*, the early man of India

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The fossilized skull of Narmada *Homo erectus* was found embedded in a conglomerate bed in the Narmada valley of central India. This bed occurs at the basal part of a formation sandwiched between two other formations of 0.73 Ma and 74000 yrs BP. The conglomerate bed has also preserved fossils of *Hippopotamus namadicus, Equus namadicus, Stegodon namadicus, Sus namadicus*, etc. having Middle Pleistocene affinity. All these stratigraphic and palaeontological evidences point to a Middle Pleistocene age of the Narmada *Homo erectus*.

A hundred years of investigation on human ancestry in India was rewarded in the year 1982 with the discovery by the first author of a skull cap of *Homo erectus* from the Quaternary alluvial deposits of the Narmada valley

in central India[1]. In its first report, a Middle Pleistocene age, was assigned to it based on a preliminary study of the associated mammalian fossils. But being the only authentic record of *Homo erectus* from the Indian subcontinent, its precise taxonomic position and age is still under discussion amongst palaeoanthropologists and palaeontologists the world over.

Subsequent multidisciplinary studies on lithostratigraphy, tephrastratigraphy, magnetostratigraphy and biostratigraphy of these deposits have thrown fresh light on the problem of age of the Narmada *Homo erectus*. Discoveries of a layer of volcanic ash and palaeomagnetic reversal in these deposits and for the first time for peninsular India are breakthroughs in fixing the chronologic position of the skull. Fresh data on the age of the Narmada *Homo erectus* are also available by additional finds of fossil mammals and a detailed taxonomic study along with a thorough review of some families of mammals.

The *Homo erectus* skull was discovered embedded (Figure 1) in a conglomerate bed within the Quaternary alluvial deposits of the Narmada Valley. Several lithostratigraphic classifications have been suggested for these deposits[2] (Table 1). The conglomerate bed occurs at the basal part of a formation designated Surajkund

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