Critical Reviews in Biochemistry and Molecular Biology, 34(6):361–385 (1999)

LDH-C_4: A Unique Target of Mammalian Spermatozoa

G. S. Gupta

Department of Biophysics, Panjab University, Chandigarh — 160014, India

I. INTRODUCTION

Lactate dehydrogenase (LDH) (L-lactate: NAD^+-oxidoreductase E.C. 1.1.1.27) exists in multimolecular tetrameric forms in a single cell. LDH has been studied widely mainly because it appears from *in vitro* studies that a considerable biochemical difference exists among various isozymes. In all higher vertebrates, the structure of somatic LDH consists two types of subunits, A and B, which are encoded by two specific genes, Ldh-a and Ldh-b, respectively. The association of A and B subunits can generate five tetrameric isozymes: A_4, A_1B_3, A_2B_2, A_3B_1, B_4, which show different catalytic properties. This in turn affects the biochemical properties of isozymes formed. Homotetramers of LDH show extreme behavior, whereas the heterotetramers show intermediate characteristics of A and B subunits (Markert et al., 1975). Because the combination of the A and B subunits can at most produce five tetrameric isozymes, the finding of the sixth isozyme in mature human testis (Blanco and Zinkham, 1963; Blanco et al., 1964) and sperm (Goldberg, 1963; 1964) indicated the presence of an additional polypeptide subunit of LDH, designated LDH-X. Zinkham et al. (1964) demonstrated LDH-X as the predominant LDH fraction in mature spermatozoa. LDH-X (also LDH-C_4) is an unique isozyme that is an iso-, allo-, and auto-antigen present only in mammalian sperm cells.

The finding of the sixth isozyme in mature human testis and sperm and loci other than A and B encoding LDH have been represented for many species (Markert et al., 1975). These loci, best characterized as 3rd Ldh locus, are observed in the testis of mammals and in some birds. The subunit was shown to be product of allelic genes (C and C′) that must be different from those that produce the A and B subunits. Evidence was obtained that this isozyme consists of a subunit that does not generally interact *in vivo* with the A and B types of LDH subunits except in pegions and swine (Blanco et al., 1964; Markert, 1963), but this interaction does occur appropriately in *in vitro* conditions (Goldberg, 1965). However, Gavella and Lipovac (1987) showed the presence of an additional form of LDH in human sperm/testis, which is a heteropolymer of B and C subunits.

The synthesis of LDH-C_4 in the testis takes place during sexual maturation, and it is the predominant lactate dehydrogenase fraction in mature spermatozoa (Zinkham et al., 1964). The catalytic properties of this

isozyme have been examined in light of the metabolic requirements of spermatogenesis as well as of mature sperm cells. Structural studies of the purified protein have established that LDH-C_4 is a distinct gene product that retains extensive homology to LDH isozymes. Biochemical studies dealing with LDH-C_4 in humans and rabbits have clearly described its properties as different from those of the other isozymes.

II. ONTOGENY AND LOCALIZATION IN TESTIS

Every mammalian species examined possesses LDH-C_4, but it is found only in the testis and spermatozoa and is not detected in any other male or female tissue. Sequence analysis revealed that mammalian LDH-C_4 is different from either the -A_4 or -B_4 more than these two isozymes are different from each other (Li et al., 1983a; Whitt, 1984). If all three genes had been evolving at the same rates, it would be reasonabe to propose that the *Ldh c* gene is the ancestral gene from which A and B genes were derived. However, sequences of -C_4 isozymes from mouse and rat are more different than would be expected. This has led to the suggestion that the -C_4 isozyme is evolving more rapidly than the -A_4 and -B_4 isozymes (Whitt, 1984). Thus, the *Ldh c* gene may have arisen after Ldh-a or Ldh-b loci and then rapidly diverged in structure.

There is a link between LDH-C_4 and spermatogenesis; prepubertal males are lacking in this enzyme and the amount of LDH-C_4 increases with testis maturity. The loss of LDH-C_4 and the regression of the seminiferous epithelium occurs after hypophysectomy of mice and rats (Goldberg and Hawtrey, 1968; review by Goldberg, 1977). At the cellular level, Hintz and Goldberg (1977) showed that midpachytene primary spermatocyte is the first stage of spermatogenesis where LDH-C_4 is detected by fluorescence intensity that increases with the progress of spermatogenesis. Thus, the Ldh-c gene is activated abruptly at a specific time in spermatogenesis. Therefore, it must be under the control of a developmental program, regulating this complex differentiation sequence. In the fox testes also LDH-C_4 first appears in pachytene spermatocytes and the localized in sperm plasma membrane (Bradley et al., 1996). Li et al. (1989) reexamined the distribution of LDH-C_4 in germ cells. The isozyme was predominant in pachytene spermatocytes, round spermatids, and condensing spermatids, whereas spermatozoa contained only LDH-C_4 isozyme. In addition, somatic LDH(s) consisting primarily of B subunit were present in germ cells throughout spermatogenesis. Sertoli cells were positive for all somatic isozymes formed by the association of A and B subunits. Burgos et al. (1995) obtained the direct evidence of a dual localization of LDH-C_4 in the cytosol of spermatocytes, spermatids, and spermatozoa, as well as in the matrix of sperm type mitochondria. Out of the total LDH-C_4 present in spermatozoa, 10% of LDH is present on sperm surface, whereas 85 and 10% are distributed in cytosol and mitochondria, respectively (Alvarej and Storey, 1984). Using the immunofluoresence technique, -C_4 has been localized on the surface of mouse and rabbit sperm (Erickson et al., 1975). Even though Takatera et al. (1993) showed that LDH-C_4 appeared in the xenografted tumor cells of human seminoma and its metastatic lesions in scid nude double mutant mice, although enzyme did not express in the original tumor of the patient. Thus, the human seminoma cells showed differential expression of the sperm specific-isozyme in parallel with their morphological changes.

III. KINETICS OF LDH-C4

LDH-C4 is different in kinetics and substrate specificity from -A4 and -B4 isozymes, and the unusual catalytic properties of testicular isozyme have been confirmed by several authors (Battelino et al., 1968; Hawtrey and Goldberg, 1970). Mouse LDH-C4 is highly thermostable and has different substrate requirements when compared with LDH-A4 and LDH-B4 (Hawtrey and Goldberg, 1970; Wheat and Goldberg, 1975; Coronel et al., 1983; Gupta and Goldberg, 1981).

A. Substrates (Metabolic Significance)

All LDH isozymes catalyze the interconversion of pyruvate and lactate, but they do so with different kinetics (Battelino et al., 1968; Coronel et al., 1983; Gupta et al., 1988). There is a compulsory sequence in interaction: first, the coenzyme is bound and then the substrate. Even allelic isozymes (B subunits) in fish have been shown to differ in kinetics and in a way that should have adaptive significance for the fish in different habitats. Ketoform of pyruvate is used as a substrate by LDH from beef heart and by pig heart LDH. Other substrates that are utilized by LDH are a number of α-hydroxy and α-keto acids, which include α-hydroxy-butyrate and α-ketobutyrate (Schatz and Segal, 1969); α-hydroxybutyrate and α-ketobutyrate have a lower V_{max} when compared with pyruvate or lactate (Figure 1).

Goldberg (1977) reviewed the substrate specificities and kinetic parameters of LDH-C_4 when compared with other LDH isozymes. LDH-C_4 has broad substrate and coenzyme specificity, particularly for α-keto acids. K_m measurements of LDH-C_4 indicated a higher affinity for lactate (Gupta et al., 1988). The turnover number for substrate transformation is much lower than for somatic LDH's. Mita and Hall (1982) showed that round spermatids from rat testes preferred lactate as an energy source

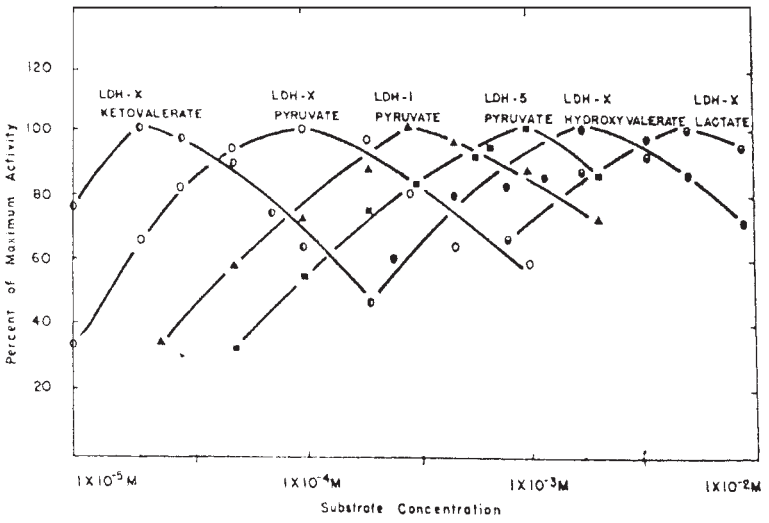

FIGURE 1. Substrate saturation curves for purified LDH isoymes. (From Hawtrey and Goldberg, 1970.)

over glucose, fructose, and pyruvate by a factor of 3 to 4. Glyoxylate can also serve as a substrate for LDH, and it can be reduced to glycolic acid as well as oxidized to oxalate (Warren, 1970). When catalytic amounts of coenzyme are present in the reaction mixture, LDH catalyzes a canizarro reaction, in that 50% of the glyoxylate is reduced and 50% is oxidized. Because LCH-C_4 replaces LDH-A and LHD-B in the testes, it shows that sperm has a special adaptive metabolism. It is thought that LDH-C_4 of spermatozoa and that present in the testicular germ cells processes lactate as a major energy source that occurs in high concentrations in rete testis fluid, oviductal fluid, and in seminal plasma. Sertoli cells do supply lactate to spermatid (Mita and Hall, 1982), and diffusion of other substrates from plasma is not adequate to support germ cells metabolism. LDH-C_4, a sperm-specific enzyme partially localized on the plasma membrane of sperm, differs in its distribution pattern compared with other somatic LDH isozymes. Sperm LDH-C_4 has been suggested to utilize lactate from seminal plasma and in the production of energy needed during sperm motility. However, Yoshida et al. (1989) showed that purified LDH-C_4 has a high affinity for pyruvate and α-ketoglutarate than lactate. Human LDH-C_4 is strongly inhibited in increasing the concentration of substrate, especially lactate. It appears that LDH-C_4 plays an important role in leucine metabolism in rat spermatogenic epithelium (Grootegoed et al., 1985). Sertoli cells convert leucine into 4 methyl–2 oxovalerate, which is converted to 2-hydroxy-4-methyl valerate by spermatocytes and spermatids. This conversion is catalyzed by LDH-C_4 in the cytosol of spermatogenic cells.

It is well known that the reaction catalyzed by LDH is inhibited in the presence of a high level of substrate; inhibition is observed in either direction, and it is more pronounced with LDH-A_4 than with LDH-B_4 (Kaplan et al., 1968). Evidence showed that phenomenon of substrate inhibition is related to the formation of ternary complex. The existence and formation *in vivo* and *in vitro* of abortive ternary complexes between LDH-NAD-pyruvate and LDH-NADH-lactate is well documented (Everse et al., 1971). LDH-C_4 is similar to the other LDH isozymes with respect to the ability to form binary or ternary complexes with associating coenzymes, susbtrates, or inhibitors (Lee et al., 1977; Gupta and Kang, 1997a). In contrast to other isozymes, there is no pH-dependent conformational change in the formation of a ternary complex.

C-DNA encoding Ldh-C was engineered in *E. coli* isopropyl β-D-thiogalactopyranoside-induced cells produced a 35-kDa subunit that spontaneously formed the enzymatically active 140-kDa tetramer. The human LDH-C_4 purified from cultured cell was heat stable and inhibited by pyruvate above 0.3 m*M*, isozyme had a Km of 0.03 m*M* for pyruvate, and a turnover number of 14000 (nmol of NADH oxidized/mol LDH/min) (Levan and Goldberg, 1991).

1. Inhibitors

The most potent inhibitor of LDH is the reduced addition compound of pyruvate and NAD^+. Reduced pyruvate adduct of (Acpy) NAD^+ is a far better inhibitor of LDH compared with a NAD^+-pyruvate adduct. Oxamate is a competitive inhibitor of LDH isozymes with pyruvate as substrate, whereas *N*-isopropyl oxamate proved to be a competitive inhibitor of LDH-C_4. Isopropylic chain conferred oxamate a high affinity for LDH-C_4 and a marked decrease in the affinity for other isozymes. Ki of LDH-1, LDH-5, and LDH-C_4 with oxamate were in the order of 0.06 m*M*, 0.08 m*M*, and 0.25 m*M*,

whereas with isopropyl oxamate Ki values were 0.04 m*M*, 0.8 m*M*, and 0.014 m*M* with three isozymes, respectively (Heck, 1969; Wong et al., 1997).

All LDH isozymes investigated to date are inhibited by sulfhydryl reagents, and this effect has been attributed to cysteine 165 (Cys–165) (Holbrook et al., 1975). Although this residue has been named the "essential thiol", it does not participate in catalysis. Rather, inhibtion occurs because the modifying agent sterically hinders coenzyme binding and catalysis. The sequence around this residue has been investigated as a dodecapeptide isolated from numerous LDH isozymes and is quite rigorously conserved (Taylor and Oxley, 1976). This peptide is of particular interest because mouse LDH-C_4 is only partially inhibited by mercurial reagents, suggesting a deletion of Cys–165 or modification around this residue (Goldberg, 1972). However, limited tryptic digestion yielded a fragment with a sequence identical to the essential thiol peptide of other mammalian LDH isozymes (Wheat et al., 1977). The result confirms the importance of this structure, but it fails to explain its role on the unusual pattern of inhibition of LDH-C_4 by mercurial agents. LDH-C_4 is less susceptible to the inhibition of catalysis by sulfhydry reagent when compared with other somatic LDHs.

Compounds that do not have the nicotinamide ring, for example, ADPR, ADP, AMP (coenzyme analogues), inhibit the reaction and are competitive with NADH. NMN^+ also binds to LDH but weakly. Because ADP, AMP, and NMN^+ all carry an additional negative charge when compared with their component parts in the coenzyme, the poor binding of these moieties in the binding of these compounds does not necessarily reflect the contribution of these moieties in the binding of NAD^+. Studies with high-resolution NMR spectroscopy indicated an interaction between chicken LDH-B_4 and the adenine moieties of 5′-AMP and ADPR (Sarma and Kaplan, 1970).

Gossypol, a polyphenolic pigment from cotton plant, is a strong inhibitor of LDH-C_4. With respect to NAD-lactate reaction, gossypol is a competitive inhibitor, whereas with respect to a NADH-pyruvate system gossypol is a noncompetitive inhibitor (Gupta et al., 1988). Among all the LDH isozymes, LDH-C_4 is the most sensitive to gossypol, and thus it forms a sensitive target for fertility regulation in males. The measurement of intrinsic fluorescence of LDH-C_4 after excitation at 280 nm revealed that gossypol quenches Trp fluorescence in a dose dependent manner; gossypol (–) being more effective than gossypol (+) and that the quenching involved two Stern-Volmer constants (Ksv) between LDH and gossypol (Gupta and Kang, 1997b).

IV. MOLECULAR CHARACTERISTICS OF LDH-C_4

Subunits of lactate dehydrogenase, including LDH-C, have been shown to have a molecular weight of about 35 kDa in all vertebrates, but the amino acid composition differs for each of the subunits and for each species. The exceptions are D-LDHs, that are dimers of 70 kDa, in some organisms and tetramers in others (Goldberg, 1977).

Pan et al. (1983) studied the 330 amino acid sequence of LDH-C_4 and compared it with LDH-A_4 and with LDH-B_4 from mouse and rat tissues. The results showed a high variation in amino acid sequence and composition among A, B, and C subunits. Li et al. (1983b) compared the amino acid sequence variation among seven LDH isozymes from dogfish muscle, chicken muscle, pig muscle and heart, mouse and rat testis, with respect to whole dehydrogenase polypeptide chains and their four functional

domains. They found that the coenzyme binding domain was more conserved than the substrate binding domain. The sequence of the loop and helix D region of testicular LDH-C_4 isozymes was very different from those of somatic LDH-A and LDH-B isozymes, while NH_2-terminal arm was extremely variable. The characteristic catalytic properties of LDH-C_4 could be attributed to the marked difference in amino acid sequence of loop helix region and coenzyme and substrate binding site of LDH-C_4 when compared with LDH-A_4 and LDH-B_4 (Figure 2).

Twenty residues in amino terminal of LDH isozyme are extremely variable, and their primary function is to stabilize the quaternary structure of tetrameric LDH through their interaction with carboxy terminal region of other subunits (Adams et al., 1973). The most parsimonious phylogenetic tree among these seven vertebrate LDH sequences clearly indicated that the LDH-A_4 and LDH-B_4 isozymes are more closely related to each other than either to the LDH-C_4 isozyme. With the use of computer graphics, space filling models of mouse and rat testes LDH-C_4 isozymes were constructed from amino acid sequence and published X-ray diffraction data. Thirty-two residues that differ between mouse and rat LDH-C sequences were also displayed on monomeric and tetrameric LDH molecules. Immunological properties of rat and mouse LDH-C_4 were compared and related to those variable 32 amino acid residues. Amino acid differences were also related to the structural-functional relationships of LDH-C_4 with somatic LDH isozymes (Li et al., 1983a).

V. 3-D STRUCTURE OF LDH-C

Positive temperature dependence and relative stability of LDH in the presence of dioxane H_2O indicated that hydrophobic forces may not be the primary forces of importance in holding the subunits together, rather coulombic attractions prevail in the stabilization of tetrameric forms (Jaenicke and Knof, 1968). Purified mouse LDH-C_4 which readily crystallizes in the presence of ammonium sulfate, formed the basis of analysis of its three-dimensional structure by X-ray diffraction. The unit cell dimensions of -C subunit are similar to the subunits of other LDH isozymes (Adams et al., 1973). At 7.5 Å resolution, the structure of this isozyme could not be distinguished from dogfish LDH-A_4 (Musick et al., 1976). When the analysis was extended to 2.9 Å resolution by the molecular replacement technique, the similarity to other LDH isozymes was even more apparent (Musick and Rossman, 1979). The overall folding of apo-LDH-C_4 polypeptide chain backbone was found to be similar to the structures of somatic LDH. The same α-helices and β-pleated sheets observed in other LDH isozymes were apparent in LDH-C_4. The unique feature of mouse LDH-C_4 structure is in the coenzyme binding loop. All other isozymes crystallized with the loop, corresponding to residues 99-112, in an open conformation (Eventoff et al., 1977). In a ternary complex with bound NAD^+ this loop moves 13 Å to cover the coenzyme. Although mouse LDH-C_4 is isolated as the apoenzyme, it crystallizes with the loop closed. If this crystal structure reflects the conformation of LDH-C_4 in solution, it could explain the low turnover number of this isozyme, that is, if the loop is in equilibrium between open and closed conformations in solution, the efficiency of coenzyme binding would be reduced (Musick and Rossmann, 1979). Triclinic crystals of apo-LDH-C_4 in 40% saturated ammonium sulfate at 4°C showed tetrameric molecule in the unit cell with the dimensions: a = 84.8 Å, b = 76.6 Å, c = 63.9 Å, = 109.7°, B = 89.5°, = 96.5° (Haogrefe et al., 1987).

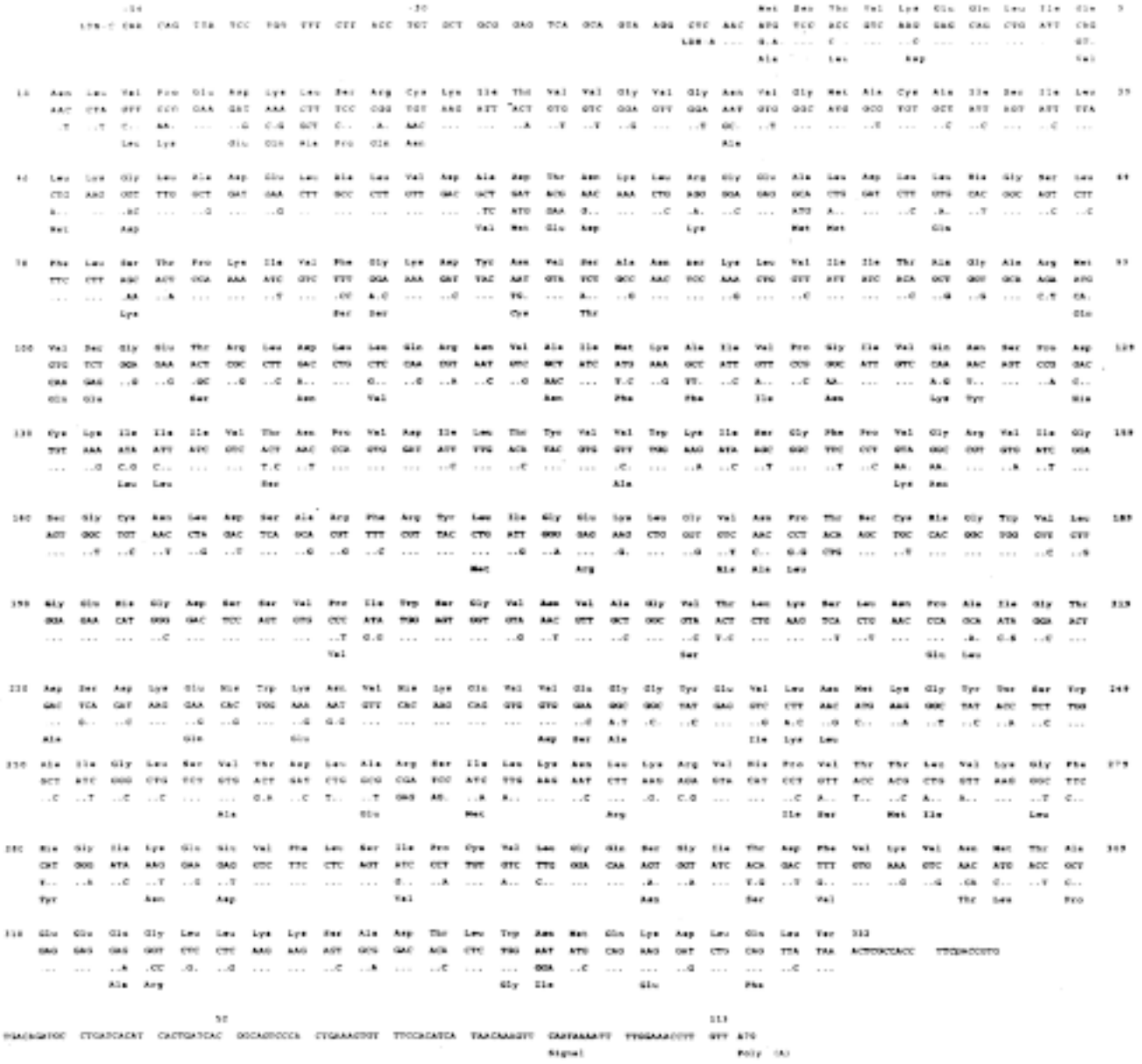

FIGURE 2. Comparison of the nucleotide and amino acid sequences of mouse LDH-C cDNA with those of mouse LDH-A. The deduced amino acid sequence of mouse LDH-C cDNA is given above its nucleotide sequence. For mouse LDH-A only those nucleotides and amino acids that differ from those of LDH-C are indicated. (From Sakai et al., 1987.)

Hogrefe et al. (1987) refined the atomic structure of mouse testicular apolactate dehydrogenase-C_4 to 3.0 Å resolution, yielding a final crystallographic factor R of 0.256. Comparison with the refined structure of dogfish apolactate dehydrogenase-A_4 showed that equivalent secondary structure elements were essentially in the same position relative to the molecular twofold axes, except in the helices: αD, αE, and α2G in the vicinity of the active center and the carboxy1 terminal helix H (Figure 3). The

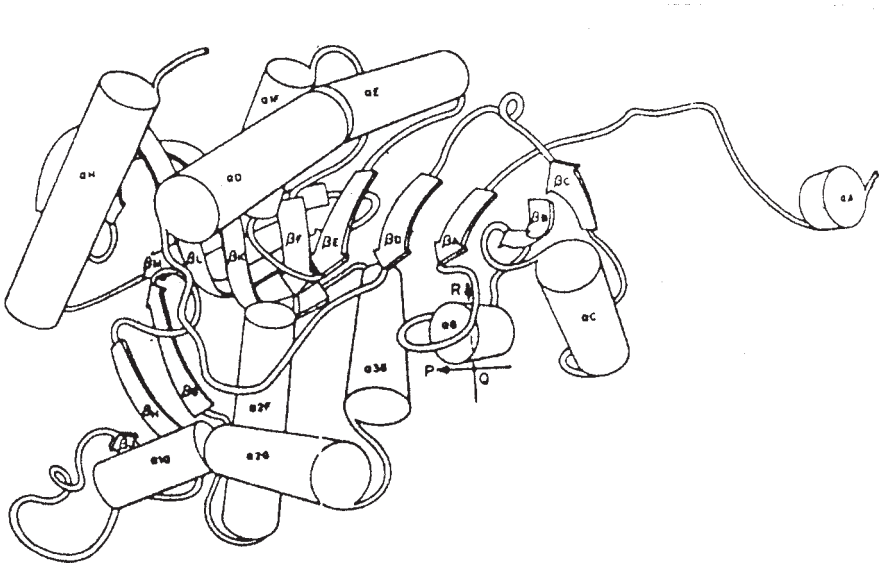

FIGURE 3. The LDH-C_4 subunit. Schematic diagram showing the secondary structure elements and their nomenclature. (From Hogrefe et al., 1987.)

immunogenic sites that elicit antibody (Ab) response are grouped into three regions; not all the epitopes are situated on the molecular surface. The portions of antigenic peptides correlate best with suretive accessibilities of the monomer rather than of the full tetrameric molecule that provides a basis for modeling of the antigen for immunocontraceptive.

A. Ldh-c c-DNA

Mouse and human sperm specific Ldh-c cDNA were cloned and sequenced from the gtλ11 expression library of Sakai et al. (1987) and Millan et al. (1987), respectively. The mouse Ldh-c cDNA insert of 1236 bp consists of the protein coding sequence (999 bp), the 5′ (54 bp), and 3′ (113 bp) noncoding region, and the poly (A) tail (70 bp). The northern blot analysis of poly (A) containing RNAs from mouse testis and liver indicated that the Ldh-c gene is expressed in testis but not in liver, and that its mRNA is approximately 1400 nucleotides in length. The nucleotide and amino acid sequence of Ldh-c cDNA of mouse showed 73 and 72% homologies, respectively, with those of the mouse LDH-A. The Southern blot analysis of genomic DNAs from mouse liver and human placenta indicated the presence of Ldh-c gene-related sequences. Studies by Wu et al. (1987) also revealed that LDH-c_4 has a 70% homology in amino acid sequence with B_4 isozyme. However, Wu et al. (1987) reported slight modifications in Ldh-c cDNA such as size comprising 1135 base pairs in length that can code 332 amino acids polypeptide and can express an mRNA of 1.5 kb in size. On the other hand, human Ldhc-cDNA comprising 1000 bp subfragment includes 66 bp of non-coding region with remaining base pairs that encodes LDH-C protein. These structural studies reveal great similarities among the LDH isozymes, confirming that LDH-A_4, LDH-B_4, and LDH-C_4 form a homologous gene family. A full-length cDNA encoding the sperm-

specific LDH was isolated from a fox testes cDNA expression library and sequenced (Bradley et al., 1996). The deduced translated protein was shown to be 86% identical to that of human LDH-C_4 with a subunit molecular weight of 35 kDa. The investigators identified an epitope within the N-terminal region of LDH-C_4 that when administered to female foxes was found to be antigenic and produced antibodies capable of recognizing the native protein (Figure 4).

VI. THE REGULATION OF Ldh-c GENE EXPRESSION IN THE TESTIS

The mouse Ldh-c gene contains a TATA box, whereas the human gene contains six potential SP1 binding sites in the same region. SP1 binds in the promoter region and appears to play a role in Ldh-c regulation at the level of transcription. The expression of Ldh-c gene is restricted to germinal epithelium and is developmentally regulated. The murine Ldh-c mRNA is first detected in preleptotene spermatocyte and persists postmeiotically to the round spermatid stage (Thomas et al., 1990). The human Ldh-c gene is interrupted by six introns at positions homolgous to those Ldh-a and Ldh-b genes (Takano and Li, 1989). The promotor of human Ldh-c has been cloned and studied in cell coculture system. In human, testis-specific expression is driven by a 180 bp fragment of promoter region (Cooker et al., 1993). Zhou and Goldberg (1996) have identified a 65-kDa protein designated as transcription initiation responsor (TIR–1) in liver nuclear extract as a possible candiate for repressor, and a 103-kDa protein designated as testis initiatiion factor (TIF–1) in testis nuclear extract with possible role in transcription initiation.

An *in vitro* transcription system was used to characterize murine Ldh-c promoter (Zhou and Goldberg, 1994; 1996). Murine Ldh-c, which is less than 25 kb away from Ldh-a on chromosome–7, arose by tandem duplication of Ldh-a. Ldh-c gene expression starts early in leptotene/zygotene spermatocytes and continues through the elongated spermatid stage of spermatogenesis. Sequence analysis of a 720-bp fragment containing promoter region revealed several ubiquitous cis-regulatory elements, including one TATA box, one GC box, and two putative GCAAT elements. Deletion analysis demonstrated that a 60-bp fragment containing a 30-bp palindromic sequence was sufficient to direct transcription in testes muclear extract (TN). This fraction was transcriptionally inactive with liver nuclear extracts (LN). Gel retardation and Southwestern assays detected different protein binding activities to this palindromic sequence in TN and LN. TATA box and 30-bp palindromic sequence were both required for promoter activity. Results suggested that palindromic sequence contains both a negative element for repression in somaic tissues and a positive element for activation of Ldh-c gene in testis (Zhou and Goldberg, 1996).

The human Ldh-c gene is a single copy gene with two alternative 5′-noncoding exons comprising an approximately 40-kb locus. The proximal region, relative to the transcription starting site, binds protein extracts from human testis but not liver. The upstream region contains three putative regulatory elements: a glucocorticoid or hormone response element (HRE), a CRE, and an octamer factor binding site (OCT). The proximal promoter contains a small CpG island that is hypermethylated in somatic tissues but undermethylated in testis (Bonny and Goldberg, 1995). Hypermethylation is indicative of transcriptional repression.

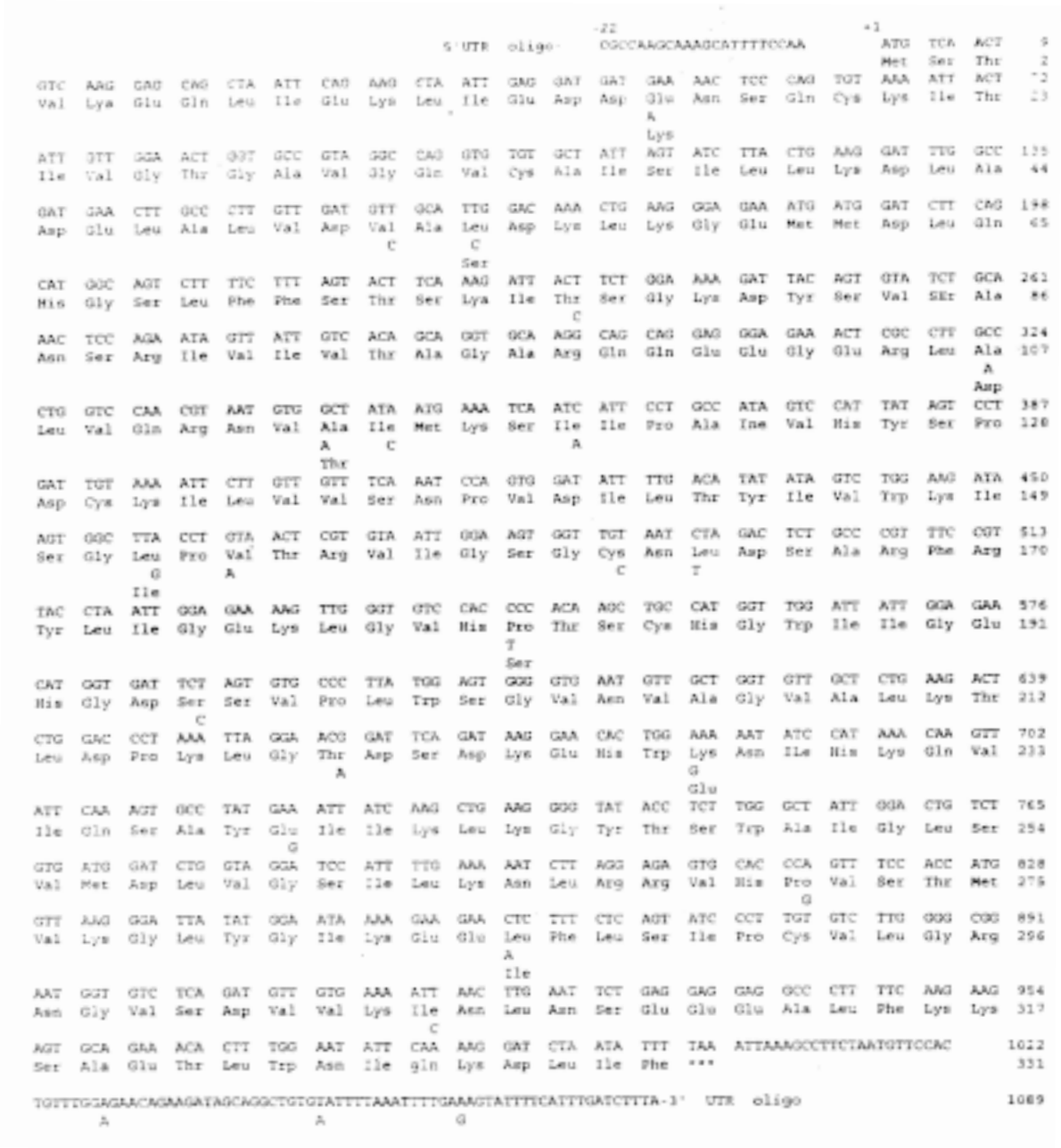

FIGURE 4. The cDNA and deduced amino acid sequences of human and baboon LDH-C cDNA. The nucleotide sequence (top line) and deduced amino acid sequence (second line) of human LDH-C are from previous studies. Only nucleotide differences (third line) and amino acid differences (fourth line) for baboon LDH-C cDNA are shown. (From O' Hern et al., 1995.)

A. c-AMP Response Element

Of all the Ldh genes, promoter activity has been rigorously demonstrated only for the rat Ldh-a gene, which has been shown to be cyclic AMP-dependent in an *in vitro* transcription system (Short et al., 1991). The mouse Ldh-c message initiates at a single transcription start site, whereas human message has multiple initiation sites

(Cooker et al., 1993). There is evidence that there are regulatory differences between the Ldh-c genes of mouse and rat, between mouse and baboon at the posttranscriptional level (Salehi-Ashtiani and Goldberg, 1993). The authors demonstrated that the Ldh-c gene message level is nearly ninefold greater in mouse testes and remains high post meiotically. In contrast, rat Ldh-c mRNA is highest in primary spermatocytes and reduced in spermatids. It was concluded that nuclear post transcriptional events contribute to the differences in Ldh-c message levels.

VII. LDH-C_4 AS AN IMMUNO-CONTRACEPTIVE

The strategy behind sperm-based contraceptives is to induce antibodies in the female reproductive tract against sperm antigen at a sufficient level to block fertilization. It is envisioned that antibodies developed by this vaccine will act before fertilization event, thus terming it as a 'prefertilization contraceptive. Being a cell-specific, LDH-C4 isozyme of spermatogenesis and spermatozoa of man and animals is partially localized on the plasma membrane of sperm (Alvarej and Storey, 1984; Erickson et al., 1975). It utilizes lactate from SP for the production of energy needed for sperm motility. These observations suggested that LDH-C4 can be used as an antigen of choice for immunocontraception in females.

A. The Immunological Distinctiveness of -C Subunit

Rabbit antibodies to highly purified crystalline LDH-C_4 from mouse do not cross-react with A and B subunits as judged by the inhibition of enzyme activity, precipitation reaction, Ouchterlony immunodiffusion, immunoelectrophoresis and complement fixation (Goldberg, 1971). Liang et al. (1986) reexamined the immunological specificity of mouse LDH-C_4 in view of report of Wright and Swafford (1984), who suggested that anti-serum to mouse LDH-C_4 is cross-reactive with LDH-A_4 and LDH-B_4. Liang et al. (1986), using enzyme linked immunoabsorbance, solid phase radioimmunoassay and competitive inhibition radioimmuno assay, demonstrated that antisera to LDH-C_4 is specific and do not cross-react with somatic isozymes, LDH-A_4, and LDH-B_4.

Binding of monoclonal antibodies (MAb) indicated that LDH-C_4 develops antibodies of high affinity ranging Ka from $1.5 \times 10^9\ M^{-1}$ to $16 \times 10^9\ M^{-1}$ (Goldman-Leikin and Goldberg, 1983). The specificity of MAb was localized to coenzyme binding loop on the basis of its reactions with synthetic peptide comprising residues 101 to 115 (Chang et al., 1979, 1980). Specificity of MAb of LDH-C_4 was further confirmed by Wang et al. (1990). Most of these Abs belonged to IgG class and appeared to be located in post-acrosome region.

In a recent study, conformational antigenic determinants in tetrameric LDH-C_4 were investigated by immunizing mice in the form of LDH complexes with substrates, coenzymes and gossypol (+) and gossypol (–) as enzyme inhibitors. Study suggested that IgG immune response is inhibited if the native enzyme was complexed with lactic acid, pyruvic acid, NAD and $NADH_2$ and gossypol (–). Maximum decline in Ab occurred when LDH-C_4 complexed with lactate or gossypol (–) was used as an antigen. Heat-denatured LDH-C_4 at 80°C for 30 min practically abolished humoral immunity, suggesting that most of the Ab response of LDH-C_4 is linked to the quaternary structure of the protein. The loss of humoral Ab

response in the presence of lactate and gossypol (–) indicated that these binding sites in LDH-C_4 are mainly involved in antigenic stimulatiion (Hundal et al., 1998, unpublished). Significantly, racemic mixture of gossypol (+) appeared to impose certain constraints that were different from those imposed by gossypol (+) or (–). Moreover, study suggested that complexed LDH-C_4 produced antibodies that were highly cross-reactive with somatic LDH isozymes. Possibly cross-reactive epitopes in LDH-C_4 are buried within the molecule, which are exposed after interaction with ligands such as gossypol and enzyme substrates (Gupta and Syal, 1999; Hundal et al., 1998) (Figure 5). Ab response generated through intrarectal route in female muce prior to mating was associated with high rate of infertility (Gupta and Syal, 1997).

B. Infertility by Immunization with LDH-C4

Studies with rodents, rabbits, and baboons have shown that the immunization of animals with purified heterologous LDH-C_4 caused a reduction of fertility but did not lead to complete infertility (Goldberg, 1973b; Goldberg, 1975b; Lerum and Goldberg, 1974; Goldberg et al., 1981; Gupta et al., 1994). Antibodies against LDH-C_4 may suppress fertility in two or more ways. The inhibition of enzyme activity by IgG(s) may impair enegy metabolism and functional mortality of spermatozoa. Alternatively, Ab binding sites on the surface of LDH-C_4 could mediate sperm agglutination as well as complement-mediated cytotoxicity (Goldberg et al., 1981). Anti-LDH-C_4 serum transferred into female reproductive tract (Kille and Goldberg, 1979) and sperm transport is markedly inhibited in female genital tract of immunized females (Kille and Goldberg, 1980). In order to determine whether immunization of both males and females would have a greater antifertility effect than immunization of one sex alone, Mahi-Brown et al. (1990) showed that the immunization of male mice but not female mice impaired fertilization. There was no significant effect on sperm transport to the oviduct either. Immunization of both sexes and subsequent mating did not have obvious synergistic effect. Gupta and Kinsky (1988, 1994) in a different enquiry, taking pregnancy as the end point, also showed that isogenic pregnancy is not affected after systemic immunization of female mice with LDH-C_4. On the other hand, a significantly higher number of viable embryos were delivered by immunized rodents. If there was any effect of LDH-C_4 on fertility rate in females, that could be seen in animals after immunization with heterologous antigen only (Gupta et al., 1994; Goldberg 1973b; Goldberg et al., 1981). Although the immunized males failed to fertilize the female mice, it appeared that male infertility was associated with testicular orchitis and autoimmune-like reactivity (Gupta and Malhotra 1994; Mahi-Brown et al., 1990). Similarly, Goldberg and Wheat (1976); and Wheat and Goldberg (1976) reported infertility following LDH-C_4 immunization in male rabbits and mice, respectively, taking pregnancy and number of offsprings as the end point but without reporting any histopathological damage of testes after immunization.

It is still not clear how immunization of males interferes with spermocyte interaction because there did not seem to be any effect on sperm transport to oviduct. Because sperm agglutination could be visualized in epididymis after immunization (Gupta and Malhotra, 1994), it is likely that lack of fertilization could be related to agglutination of sperm after interaction with antibodies. Nonetheless, antibody-mediated complement lysis of sperm cannot be ruled

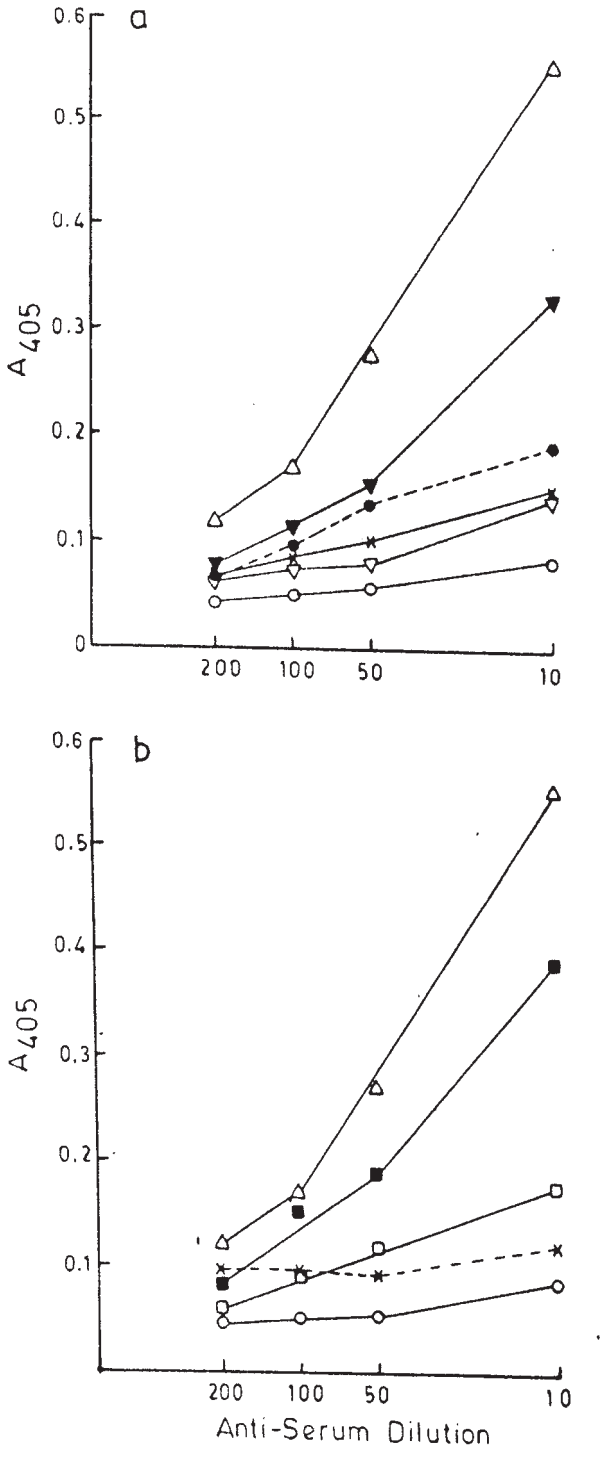

FIGURE 5. ELISA titer curves analyzed by indirect method. Absorbance at 405 nm (A_{405}) for antibody response in sera produced after immunization of female Balb/c mice with native LDH-C_4 from testes of LACA mice through intrarectal route ($1 \times 50 + 2 \times 30$) μg as positive control (△–△) and LDH complexes with (a) lactic acid (▼–▼), pyruvic acid (▽–▽), NAD(×–×), NADH (●–●); (b) gossypol (–) (■–■), gossypol (+) (□–□), and heat denatured at 80°C for 30 min (×---×). Nonimmune serum (○–○) served as negative control. Plates were coated with 0.1 μg LDH-C_4. (From Hundal et al., 1998; unpublished data.)

out (Goldberg et al., 1981). Thus, it was suggested that it would be prudent to pursue LDH-C_4 as an antigen for female vaccination unless its safety is established in males (Mahi-Brown et al., 1990).

To define the basis of LDH-C4 immunity in relation to contraception in females, cell-mediated cytotoxic responses to LDH-C_4 were tested by ^{51}Cr release assay. Lysis of labeled tumour cells by LDH-C_4 immunized splenocytes supported the cytolysis of sperm in the female reproductive tract as one of the mechanisms whereby fertility is reduced following immunization with LDH-C_4 (Shelton and Goldberg, 1985a; 1985b). In a subsequent report Shelton and Goldberg (1986) suggested the cause of reduction of fertility in females to be associated with the induction of local mucosal immunity and the release of the IgA class of antibodies specific to LDH-C_4. On the other hand, it has been reported that -C4 is immunosuppressive for alloimmune responses, including CTL, and that immunization with sperm-specific LDH generates suppressor T-cells *in situ* (Gupta and Kinsky, 1993; Gupta et al., 1992). Besides, the effect is more significant with female splenocytes when compared with male spleen cells (Gupta et al., 1996). However, the suppressive effect of LDH-C_4 on the B cell function was identical on both males and females. The role of LDH-C_4 in the protection of allogenic sperm in the female genital tract has also been suggested (Gupta and Kinsky, 1994).

In contrast to native LDH-C_4, the chemically modified allogenic LDH-C4 in the form of glucosylated protein (Glu-LDH-C_4) and in the form of protein-gossypol adduct (Gossy-LDH-C_4) were highly immunogenic for IgG immune response and were most effective in reducing fertility after inoculation through intra-rectal route using $Al(OH)_3$ as an adjuvant (Gupta and Syal, 1997). It appeared that infertility induced by modified LDH-C_4 was due to a change in conformation and exposure of cross-reactive antigenic epitopes that were more immunogenic for Ab response (Gupta and Syal, 1999). Significantly, racemic mixture of gossypol (+) gave higher antibody titer than the titer produced by gossypol (–), which was more immunogenic than gossypol (+). It indicated that stereospecificity of gossypol plays a significant role in inducing Ab and infertility (Gupta and Kang, 1997b; Hundal et al., 1998) (Figure 5). It appeared that gossypol induces infertility in males as a toxic manifestation of the gossypol-LDH complex (Gupta and Syal, 1999).

In another approach, Adoyo et al. (1995) identified components from guinea pig acrosomes that might have been conserved during evolution can be used for development of an immunocontraceptive. In one clone (1.75 kb), the first 1132 nucleotides displayed 90% homology to human LDH-C_4 gene. The finding suggests that a strategy involving the screening across species for conserved moieties of mammalian acrosome may be useful for identifying antigens for a immuno contraceptive.

C. The Regulation of Immune Response: Effect of Adjuvant and Route of Immunization

The genetic control of immune response to mouse LDH-C_4 has been investigated in mice (Kille et al., 1978). Because athymic nude mice do not respond, LDH-C_4 must be a T-dependent antigen. A cellular response to -C_4 was demonstrated by a delayed-type hypersensitivity reaction. Magnitude and kinetics of cellular immune reponse appeared to be under separate genetic control, none of which was associated with H-2 complex or with Ig allotype. This type of response by LDH-C_4 was unusual in the sense that Ab to

LDH-B_4 as well as many other protein antigens is controlled by Ir genes in the H-2 complex (Melchers et al., 1973).

The effect of LDH-C_4 alone or with MDP and FCA on immune respones and breedng capacity in isogenic mice was studied after systemic immunization (Gupta and Kinsky, 1994). The amplication of MDP as a adjuvant in IgG response was half of that induced by FCA. Systemic immunization (s.c.) did not reduce the overall pregnancy rate but reduced the frequency of embryo resorption and increased litter size significantly. Local graft vs. host reaction (LGVH) revealed that maternal lymphocyte cell competence was depressed by LDH-C_4 during gestation. MDP, like LDH-C_4 alone, did not modify LGVH reaction significantly, although it exerted an effect similar to LDH-C_4 in embryo protection (Gupta and Kinsky, 1994). However, $Al(OH)_3$ used as an adjuvant through intrarectal route appeared to induce a faily good Ab response, that was not associated with any immunological suppression of lymphocyte proliferation *in vitro* (Gupta and Syal, 1997).

In order to improve the secretory immune response in the uterine tract, Alexander et al. (1992) exposed the uterei of mice directly to LDH-C_4 with and without various combinations of adjuvants as muramyl dipeptide (MDP) and cholera toxin B (CTB). Measurable antibodies (IgA and IgG) in uterine flushings were not detected unless primary systemic immunization was employed. MDP and CTB helped in enhanced antibody response. When different sites and sequence of immunization was compared uterine Ab levels were highest when systemic antigen exposure was followed by a gastric exposure and then an intrauterine challenge. Female rhesus macaques developed circulating antibodies following primary s.c. immunization of LDH-C_4 with *Bordetella pertussis* followed by boosters after a week in the presence of aerosol to stimulate BALT or by enterically coated capsules to stimulate GALT. Immune challeges consisted of antigen administered intravaginally. Levels of IgA and IgG in vaginal washes did not mirror serum level, indicating local production rather than transduction of these immunoglobulins. Thus in both animal models local IgA production in reproductive tract required systemic priming followed by local secondary immunization of LDH-C_4.

D. Immunological Protection of Pregnancy by LDH-C_4

Effects on fertility of female mice after hyper-immunization with human LDH-C_4 have been studied for two consecutive cycles of pregnancy (Gupta et al., 1994). Taking pregnancy as the end point, low dose of antigen produced higher Ab response and higher infertility (70%) than that produced by higher dose of LDH-C_4 (46%) after first cycle. However, fertility and antibody response before mating after two doses were reversed during second cycle of pregnancy. LDH-C_4-induced infertility was reversible. In a significant observation, the fertile immunized dams delivered a significantly higher litter size when compared with nonimmunized control dams. It was concluded that immunity due to LDH-C_4 is beneficial to pregnancy for embryo survival as substantiated in another trial reported elsewhere (Gupta and Kinsky, 1994). It has been suggested that a low level of LDH-C_4 is immunosuppressive for cellular immune responses against hiscompatibility antigen, and prior immunization of virgin mice with LDH-C_4 decreases the pregnancy of embryo resorption in syngenic pregnancy. Prior immunization with LDH-C_4 not only suppresses local graft vs. host reaction, but also induces formation of suppressor T cells and

generation of cytotoxic T cells *in vitro* (Gupta and Kinsky 1993, Gupta et al., 1992). LGVH reactivity in hybrid mice also indicated a suppression of stimulation index in primary and secondary set of LGVH in LDH-C_4-sensitized mice, whereas rejection of allograft of SaI (A/J) in C_{57} Bl/Ks mice in the presence of LDH-C_4 was significantly delayed. LDH-sensitized lymphocytes from female mice were more prone to immune suppression by LDH-C_4 than female lymphocytes (Gupta et al., 1996; Gupta and Chaturvedi, 1999).

VIII. LDH-C_4 BASED PEPTIDE IMMUNOCONTRACEPTIVE VACCINE

The antigenic structure of LDH-C for the development of an immunocontraceptic has been studied (Wheat and Goldberg 1985a,b; Wheat et al., 1985; Beyler et al., 1985; Gonzales-Prevatt et al., 1982). The replacement of the natural isozyme with a synthetic antigen would ensure the availability and homogenity of vaccine preparations as well as guarantee the antigenic specificity of LDH-C_4 (Goldberg et al., 1983). These short peptides coupled to carrier molecules can generate antibodies against molecule from which peptides are derived. Wheat and Goldberg (1983; 1985a) and Beyler et al. (1985) identified tryptic peptides of mouse LDH-C_4 that bind Ab against the native protein. The most antigenic peptides are relatively nonhydrophilic. Because antigenic domains after contain proline residue, peptides containing antigenic domains include 8 of a total of 11 proline residues of LDH-C_4. The association of proline with antigenic domains is probably a function of the effect of that residue on the fold of polypeptide chain, inducing reverse turns rather than some special chemical interactions with the immune system. One pure peptide that represents residues 152 to 159 of mouse LDH-C_4 was active in binding anti-LDH-C_4 and its synthetic analogue was tested to induce an immune response to native protein (Gonzales-Prevatt et al., 1982). Based on the presence of antigenic sequences on the surface of LDH-C_4 (Hogrefe et al., 1987), it was possible to study immunological properties of a panel of synthetic peptides that represent the most accessible and mobile segments of LDH-C (Hogrefe et al., 1989). Ten peptides corresponding to mouse LDH-C amino acid sequences: 1-14, 5-15, 49-58, 97-110, 211-220, 231-243, 274-286, 304-316, and 318-330 were synthesized and compared in terms of binding of antibodies raised in rabbits against native intact protein. Immunogenicity of mouse LDH-C peptides coupled to diphthreia Toxoid (DT) in rabbits could be ranked in following order: 5-15, 304-316 > 211-220, 274-286 > 49-58, 97-110; the antigenicity of best peptides was 1/8th of the whole structure of LDH-C_4 (Figure 6).

Three synthetic peptides based on residue no. 310-327 of mouse LDH-C_4, and their conformations were evaluated for immunogenicity. An 18-residue random coil (αN) and its amphipathic alpha helix (α1) were weakly immunogenic. A conformationally stable 40 residue alpha-alpha-fold peptide (α3) was highly immunogenic and reacted with a native molecule by ELISA (O'Hern, 1991).

Kaumaya et al. (1990, 1992, 1993) designed, synthesized, and characterized model peptides with predetermined secondary, tertiary, and quaternary structures in an attempt to mimic the three-dimensional structure of an antigenic determinant of LDH-C_4. Kaumaya et al. (1992) demonstrated that a putative topographic determinant of the testis-specific isozyme of LDH-C_4, designed and synthesized to adopt a well-defined alpha-helical secondary and tertiary structure

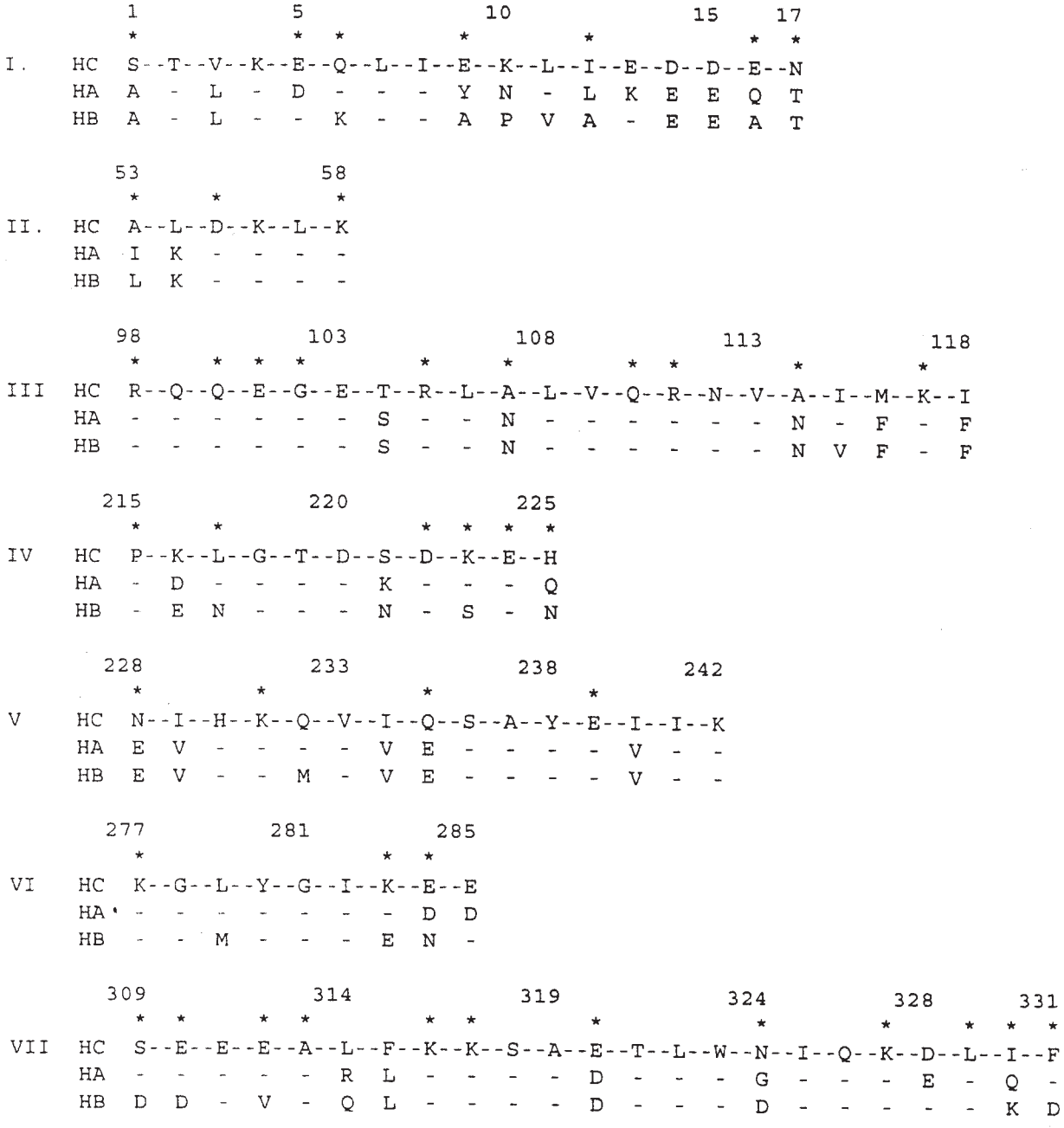

FIGURE 6. Amino acid sequence variability in the putative epitopes of human LDH-C_4. The seven most accessible segments of the mouse LDH-C_4 molecule (Hogrefe et al., 1987) are shown, with the corresponding amino acid sequences of human LDH-C_4 (HC) human LDH-A_4 (HA) and human LDH-B_4 (HB). Asterisks mark the positions of residues that are particularly accessible to a 10-A radius probe in mouse LDH-C_4. (From Hogrefe et al., 1989.)

(four helix bundle motif) in aqueous solutions, is highly immunogenic in rabbits and mice, inducing IgG antibodies that bind to native LDH-C_4. This engineered conformational 40-residue peptide was shown to be considerably more effective in inducing anti-

bodies, when compared with the corresponding linear peptide. The antibody response that was obtained without coupling the peptide to a carrier protein suggested that the peptide contains a T-cell antigenic determinant.

An ideal peptide vaccine should contain both B- and T-cell epitopes. The strategies for co-inclusion of T-cell 'helper' epitopes with the B-cell determinant eliciting immune responses that are in most cases genetically restricted to only one or few alleles of the MHC with limited activity across divergent MHC class II haplotypes. This genetically restricted T-cell costimulatory activity of peptides is a serious obstacle, and consequently such constructs would be of limited practical value as a vaccine targeted to a majority of an outbred population. Kaumaya et al. (1993) constructed a contraceptive peptide vaccine and engineered two peptides to encompass the sequences from the universally immunogenic tetanus toxoid (TT) epitope and the contraceptive vaccine candidate LDH-C_4. It was demonstrated that the peptide vaccine containing a 'promiscuous' T-cell epitopes and a defined B-cell epitope was able to induce high titer antipeptide IgG antibodies specific for native protein antigen LDH-C_4 in several inbred strains of mice, outbred mice, and rabbits.

Kobs-Conrad et al. (1993) showed that an antigenic epitope from the model contraceptive vaccine candidate LDH-C can be rationally engineered into a highly structured conformation that mimics the corresponding site in the native three-dimensional protein. It was also suggested that the antibodies raised to the various construct had high affinity for native protein.

The conformations of designed antigenic peptides that mimic to various degrees the antibody binding regions have been investigated by deuterium hydrogen exchange and electropray ionization mass spectrometry (ESI-MS). The technique described a viable method for the characterization of the conformations of protein antigen such as LDH-C_4 and for screening the conformation of antigenic peptides designed to elicit optimal immune responses (Downard, 1997).

Antigenic properties of LDH-C_4 of pig, rabbit, and rat as well as of human LDH-C_4 were studied using antipeptide antibodies against pig LDH-M_4 fragment (180–214) (Grebenshchikova and Prozorovski, 1991). Authors identified an amino acid sequence 180–214 containing His-195 that was involved on the active site of LDH isozymes. It proved to be a total antigenic determinant only for human and pig M4-isozymes. An amino acid sequence of total antigenic determinant did not allow any substitution of essential amino acid residues. His–195, participating in substrate binding, was not involved immediately in reactions with antibodies as shown by means of chemical modification of pig M4-isozyme with diethyl pyrocarbonate and after production of antibodies against the modified isoenzyme.

O'Hern et al. (1995) reported that the first 20 residues of the amino terminal arm were likely to contain the most immunogenic portion of human LDH-C. Out of this sequence residue 9 to 20 (C9–20) conjugated to diphtheria toxoid was the best antigen in eliciting Ab response over other over lapping antigens. DT-C9–20 was tested in female baboons for immunological infertility. In a trial of 14 females 12 were infertile in immuniozed group compared with 7 pregnant baboons out of 15 in nonimmunized control after 4 menstrual cycles. However, in reversal trial after 1 year, the pregnancy rate in the immunized group was not different from the control baboons. There was no correlation between Ab titer and infertility.

IX. LDH-C_4 AS A MARKER

A. Testis Injury and Infertility

Pathological disturbances of spermatogenesis result in disturbance of LDH-C_4. The isozyme is not detected in seminal plasma or testicular biopsies from infertile men showing azospermia or aspermia (Szeinberg et al., 1966). In addition, isozyme is absent from cryptorchid testis (Goldberg and Hawtrey, 1968) and in radiation-induced atrophic testis (Gupta and Bawa, 1981). LDH-C_4 has been found to be absent from the semen of men with azosermia, and it also disappears after vasectomy (Eliasson et al., 1967; Prasad et al., 1976; Keltimlidis et al., 1988). Virji and Eliasson (1985) showed that LDH-C_4/sperm ratio can be related to the status of seminiferous epithelium.

As an infertility index in men, Gavella and Cvitkovi (1985) and Velasco et al. (1993) determined LDH-C_4 in seminal fluid. It appeared that LDH-C_4 discriminates well between infertile groups with oligospermia and normo-spermic subjects and between infertile groups and fertile group with proven fertility. It was concluded that LDH-C_4 offers a clinical utility marker for both germinal activity and spermatozoid quality. However, Laudat et al. (1997) did not find a correlation between seminal LDH-C_4 and sperm motility in patients with acrosomal abnormalities. Occasionally, LDH-C_4 increases in the serum of men after testis injury following exposure of toxic substances such as muscle toxicant 2,3,5,6 tetramethyl *p*-phenylene diamine (TMPO) (Draper et al., 1994), 1, 3-dinitrobenzene (DNB) and ethylene glycol monomethyl ether (EGME) (Reader et al., 1991). In some cases LDH-C_4 actvity correlated with androgen binding protein in blood plasma (Reader et al., 1991).

A. In Forensic Science

The usefulness of LDH-C_4 as a semen-specific marker in blood-semen mixture has been tested using various techniques. Polyclonal antisera of LDH-C_4 were highly sensitive and highly specific (Pawlowski and Brinkmann, 1992).

ACKNOWLEDGMENTS

This work was supported by the Indian Council of Medical Research and the Council of Scientific and Industrial Research, New Delhi.

REFERENCES

Adams AD, Adams MJ, Rossman MG, and Goldberg E. 1973. A crystalline form of testes specific lactate dehydrogenase. *J Mol Biol* **78:** 721–722.

Adoyo PA, Moore A, and Moore HD. 1995. A strategy for indentifying candidate sperm antigens for immunocontraception: Isolation of human testis cDNA clones using polyclonal antisera directed against hamster acrosomal membrane preparation. *Int J Androl* **18:** 32–38.

Alexander NC, Falgham DL, and Goldberg E. 1992. Contraceptive Vaccine Development: Secretory Immune response in mice and monkeys. *Vaccine Res* **1:** 331–346.

Alvarej JG and Storey BT. 1984. Assessment of cell damage caused by spontaneous lipid peroxidation in rabbit spermatozoa. *Biol Reprod* **30:** 323–331.

Battelino LJ, Jaime FR, and Blanco A. 1968. Kinetic properties of rabbit testicular lactate dehydrogenase isozyme. *J Biol Chem* **243:** 5158–5192.

Beyler SA, Wheat TE, and Goldberg E. 1985. Binding of antibodies against antigenic domains of Murine lactate dehydrogenase-C_4 to human and mouse spermatozoan. *Biol Reprod* **32:** 1201–1210.

Blanco A and Zinkham W H. 1963. Lactate dehydrogenase in human testis. *Science* **139:** 601–602.

Blanco A, Zinkham WH, and Kupchyk L. 1964. Genetic control and ontogeny of lactate dehydrogenase in pigeon testis. *J Exp Zool* **156:** 137–152.

Bonny C and Goldberg E. 1995. The CpG-rich promoter of human LDH-C is differently methylated in expressing and non-expressing tissues. *Dev Genet* **16:** 210–217.

Bradley M, Geelan A, Leitch V, and Goldberg E. 1996. Cloning, sequencing and characterization of LDH-C_4 from a fox testes cDNA library. *Mol Reprod Dev* **44:** 452–459.

Burgos C, Maldonado C, Burgos G de NN, Aoki A, and Blanco A. 1995. Intracellular localization of the testicular and sperm specific lactate dehydrogenase isozyme-C_4 in mice. *Biol Reprod* **53:** 84–92.

Chang SMT, Lee CY, and Li SS-L. 1979. Structural relatedness of mouse LDH isozymes A_4. muscle), B_4 (heart) and C_4 (testis). *Biochem Genet* **17:** 715–729.

Chang SMT, Lee CY, and Li SS-L. 1980. Some chemical properties of rat testicular lactate dehydrogenase. *Int J Biochem* **11:** 1–6.

Cooker LA, Brooke CD, Kumari M, Hofmann M-C, Millan JL, and Goldberg E. 1993. Genomic structure of promoter activity of the human testis lactate dehydrogenase gene. *Biol Reprod* **48:** 1309–1319.

Coronel CE, Burgos C, Burgos NNGD, Rovai LE, and Blanco A. 1983. Catalytic Properties of sperm specific lactate dehydrogenase (LDH-X or C_4) from different species. *J Exp Zool* **225:** 379–385.

Draper RP, Waterfield CJ, York MJ, and Timbrell JA. 1994. Studies on muscle toxicant 2,3,5,6-tetramethyl *p*-phenylene-diamine: effects on various biomarkers including urinary creatine and taurine. *Arch Toxicol* **69:** 111–117.

Downard KM. 1997. Characterization of the conformations of antigenic peptides of protein lactate dehydrogenase (LDH-C_4) by electro spray ionization mass spectrometry. *Mass Spectrom* **11:** 1853–1858.

Eliasson R, Haggman K, and Wiklung B. 1967. Lactate dehydrogenase in human seminal plasma. *Scand J Clin Lab Invest* **20:** 353–358.

Erickson RP, Friend DS, and Teinenbaum D. 1975. Localization of mouse LDH-X on the surface of mouse spermatozoa. *Exp Cell Res* **91:** 1–5.

Eventoff W, Rossman MG, Taylor SS, Torff HJ, Meyer H, Keil W, and Kittz HH. 1977. Structural adaptations of lactate dehydrogenase isozymes. *Proc Natl Acad Sci USA* **74:** 2677–2681.

Everse J, Barnett RE, Thorne CJR, and Kaplan NO. 1971. The formation of ternary complexes by diphosphopyridine nucleotide dependent dehydrogenases. *Arch Biochem Biophys* **143:** 444–460.

Gavella M and Cvitkovi CP. 1985. Semen LDH-X deficiency and male infertility. *Arch Androl* **15:** 173–176.

Gavella M and Lipovac V. 1987. A study of additional forms of the human sperm lactate dehydrogenase. *J Reprod Fert* **81:** 391–596.

Goldberg E. 1963. Lactic and malic dehydrogenases in human spermatozoa. *Science* **139:** 601–602.

Goldberg E. 1964. Lactate dehydrogenases and malate dehydrogenases in sperm. Studies by polyacrylamide gel electrophoresis. *Ann NY Acad Sci* **127:** 560–570.

Goldberg E. 1965. Lactate dehydrogenases in spermatozoa: Subunit interactions *in vitro. Arch Biochem Biophys* **109:** 134–141.

Goldberg E. 1971. Immunochemical specificity of lactate dehydrogenase-X. *Proc Natl Acad Sci USA* **68:** 349–352.

Goldberg E. 1972. Amino acid composition and properties of crystalline lactate dehydrogenase-

X from mouse testes. *J Biol Chem* **247:** 2044–2048.

Goldberg E. 1973. Infertility in female rabbits immunized with LDH-C_4. *Science* **181:** 458.

Goldberg E. 1975. Effects of Immunization with LDH-X on fertility. *Acta Endocrinol* **78:** 318–323.

Goldberg E. 1977. Isozymes in testes and spermatozoa, In: *Isozymes: Current Topics in Biological and Medical Research.* Ratazzi MS, Scandalios JG, and Whitt GS, Eds. New York, Alan R Liss, Vol. 1, pp. 79–124.

Goldberg E and Hawtrey C. 1968. The effect of experimental cryptorchidism on the isozymes of lactate dehydrogenase in mouse testes. *J Exp Zool* **167:** 411–418.

Goldberg E and Wheat TE. 1976. Induction of infertility in male rabbits by immunization with LDH-X. In: *Regulatory Mechanisms of Male Reproductive Physiology*. Spilman CH, Labl TJ, and Kriton KT, Eds. Amsterdam. Excerpta Medica, pp. 133–138.

Goldberg E, Wheat TE, Powell JE, and Stevens VC. 1981. Reduction of fertility in female baboons immunized with lactate dehydrogenase-C_4. *Fertil Steril* **35:** 214–217.

Goldberg E, Wheat TE, and Shelton JA. 1983. Sperm specific LDH and development of a synthetic contraceptive vaccine. In: *Reproductive Immunology*. Isojima S and Willington WD, Eds. Elsevier Science Publishers, New York, pp. 215–223.

Goldman-Leikin RE and Goldberg E. 1983. Characterization of monoclonal antibodies to the sperm specific lactate dehydrogenase isozyme. *Proc Natl Acad Sci USA* **80:** 3774–3778.

Gonzales-Prevatt V, Wheat TE, and Goldberg E. 1982. Identification of an antigenic determinant of lactate dehydrogenase-C_4. *Mol Immunol* **19:** 1579–1585.

Grebenshchikova OG and Prozorovski VN. 1991. Antigenic properties of lactate dehydrogenase isozymes of various biological types using antipeptide antibodies to a fragment of the active center of swine lactate dehydrogenase-M_4. *Vopr Med Khim* **37:** 21–24.

Grootegoed JA, Jansen R, and Vander-Molen HJ. 1985. Intracellular pathway of leucine catabolism in rat spermatogenic epithelium. *Biochem J* **226:** 889–892.

Gupta GS and Bawa SR. 1979. Radiation effects on testes. XV. Role of NAD and NADP linked dehydrogenases in radiation induced atrophy. *Strahlentherapie* **155:** 287–292.

Gupta GS and Chaturvedi G. 1999. Regulation of immune functions by sperm specific LDH and its differences with somatic isozyme in primary and secondary lymphocyte cultures. *Am J Reprod Immunol* (communicated).

Gupta GS, Chaturvedi G, and Joshi A. 1996. Sex-dependent immunological suppression by LDH isozymes. *Mol Cell Biochem* **158:** 115–119.

Gupta GS and Goldberg E. 1981. Thermodynamic stability of lactate dehydrogenase isozymes from rabbit and guinea pig. *Ind J Biochem Biophy* **18:** 182–186.

Gupta GS and Kang BPS. 1997a. LDH-C_4 substrate binary complexes studied by intrinsic fluorescence method. *Ind J Biochem Biophys* **34:** 307–312.

Gupta GS and Kang BPS. 1977b. Quenching of intrinsic fluorescence of sperm specific LDH by optical isomers of gossypol. *Gen Physiol Biophys* **16:** 215–225.

Gupta GS, Kapur S, and Kinsky RG. 1988. Inhibition kinetics of lactate dehydrogenase isozymes by gossypol acetic acid. *Biochem Int* **17:** 25–34.

Gupta GS and Kinsky RG. 1988. Immunoregulatory properties of lactate dehydrogenase-C_4 *in vivo* and its effect on embryo resorption. *Hum Reprod* **3:** 120.

Gupta GS and Kinsky RG. 1993. Modulation of alloimmune responses *in vivo* and *in vitro* by sperm-specific lactate dehydrogenase-C_4. *Mol Cell Biochem* **125:** 145–151.

Gupta GS and Kinsky RG. 1994. Effects of Immunization with sperm specific lactate dehydrogenase with and without muramyl dipeptide as adjuvant. *Ind J Med Res* **100:** 98–105.

Gutpa GS, Kinsky RG, and Couderc J. 1992. Modulation of T-cell function by sperm-specific lac-

tate dehydrogenase: Fluorescence analysis of immune competent cells and local graft versus host reaction. *Ind J Biohem Biophys* **29:** 271–276.

Gupta GS and Malhotra R. 1994. Autoimmune-like activity of sperm specific LDH: A pathophysiological and electron microscopic study of atrophied testis and epididymis. *Ind J Biochem Biophys* **31:** 480–485.

Gupta GS, Malhotra R, and Sehgal S. 1994. Regulation of fertility in female mice after immunization with human sperm specific LDH: role in conception and contraception. *Ind J Exp Biol* **32:** 14–19.

Gupta GS and Syal N. 1997. Immune responses of chemically modified homologous LDH-C_4 and their effects on fertility regulation in mice. *Am J Reprod Immunol* **37:** 206–211.

Gupta GS and Syal N. 1999. Immunologically cross-reactive epitopes in sperm specific LD: immunological consequences after glucosylation and gossypol interaction. *J Androl* (communicated).

Hundal MK, Syal N, and Gupta GS. 1998. Immune responses of chemically modified LDH-C_4. *Am J Reprod Immunol* **40:** 259.

Hawtrey CO and Goldberg E. 1970. Some kinetic aspects of sperm-specific lactate dehydrogenase in mice. *J Exp Zool* **174:** 451–462.

Heck H d'a. 1969. Porcine heart lactate dehydrogenase. Optical rotatory dispersion, thermodynamics and kinetics of binding reactions. *J Biol Chem* **244:** 4375-

Hintz M and Goldberg E. 1977. Immunohistochemical localization of LDH-X during spermatogenesis in mouse testes. *Dev Biol* **57:** 375–384.

Hogrefe HH, Griffith JP, Rossmann, MG, and Goldberg E. 1987. Characterization of the antigenic sites on the refined 3 Å resolution structure of Mouse Testicular LDH-C_4. *J Biol Chem* **262:** 13155–13164.

Hogrefe HH, Kaumaya PT, and Goldberg E. 1989. Immunogenicity of synthetic peptides corresponding to flexible and antibody-accessible segments of mouse lactate dehydrogenase (LDH)-C_4. *J Biol Chem* **264:** 10513–10519.

Holbrook JJ, Lilgas A, Steindel SJ, and Rossman MJ. 1975. Lactate dehydrogenase. In: *The Enzymes.* Vol. II, Boyer PD, Ed. New York, Academic Press, p. 191–292.

Jaenicke R and Knof S. 1968. Molecular weight and quaternary structure of lactic dehydrogenase: comparative determination of sedimentation analysis, light scattering and osmosis. *Eur J Biochem* **4:** 157–162.

Kaplan NO, Everse J, and Admiraal J. 1968. Significance of substrate inhibition of dehydrogenases. *Ann NY Acad Sci* **151:** 400–411.

Kaumaya PT, Berndt KD, Heidorn DB, Trewhella J, Kezdy FJ, and Goldberg E. 1990. Synthesis and biophysical characterization of engineered topographic immunogenic determinants with alpha alpha topology. *Biochemistry* **29:** 13–23.

Kaumaya PT, VanBuskirk AM, Goldberg E, and Pierce SK. 1992. Design and immunological properties of topographic immunogenic determinants of a protein antigen (LDH-$_4$) as vaccines. *J Biol Chem* **267:** 6338–6346.

Kaumaya PT, Kobs-Canrad S, Seo YH, Lee H, VanBuskirk AM, Feng N, Sheridan JF, and Stevens V. 1993. Peptide vaccines incorporating a 'promisuous' T cell epitope bypass certain haplotype restricted immune responses and provide broad spectrum immunogenicity. *J Mol Recognit* **6:** 81–94.

Keltimlidis K, Papadimas J, Bontis J, and Mantalenakis S. 1988. LDH isozymes in semen of infertile men. *Arch Androl* **22:** 77–84.

Kille JW and Goldberg E. 1979. Female reproductive tract immunoglobulin responses to a purified sperm-specific antigen (LDH-C_4). *Biol Reprod* **20:** 863–871.

Kille JW and Goldberg E. 1980. Inhibition of oviductal sperm transport in rabbits immunized against sperm-specific lactate dehydrogenase (LDH-C_4). *J Reprod Immunol* **2:** 15–21.

Kille JW, Wheat TE, Mitchell G, and Goldberg E. 1978. Strain differences in the immune response of mice to homologus sperm-specific lactate dehydrogenase-C_4. *J Exp Zool* **204:** 259–265.

Kobs-Conrad S, Lee H, DiGeorge AM, and Kaumaya PT. 1993. Engineered topographic determinants with alpha beta, beta alpha beta and beta alpha beta alpha topologies show high affinity binding to native protein antigen (lactate dehydrogenase-C_4). *J Biol Chem* **268:** 25285–25295.

Laudat A, Foucault P, and Palluel AM. 1997. Relationship between seminal LDH-C_4 and spermatozoa with acrosome anomalies. *Clin Chim Acta* **265:** 219–224.

Lee CY, Pegoraro B, Topping JL, and Yuan JH. 1977. Purification and biochemical studies of lactate dehydrogenase-X from mouse. *Mol Cell Biochem* **18:** 49–57.

Lerum JE and Goldberg E. 1974. Immunological impairment of pregnancy in mice by lactate dehydrogenase-X. *Biol Reprod* **11:** 108–115.

Levan KM and Goldberg E. 1991. Properties of human testis-specific lactate dehydrogenase expressed from *Escherchia coli. Biochem J* **273:** 587–592.

Li SS-L, Feldmann RJ, Okabe M, and Pan Y-CE. 1983a. Molecular features and immunological properties of lactate dehydrogenase C_4 isozymes from mouse and rat testes. *J Biol Chem* **258:** 7017–7028.

Li SS-L, Fitch WM, Pan Y-CE, and Sharief FS. 1983b. Evolutionary relationships of vertebrate lactate dehydrogenase isozymes A_4 (muscle), B_4 (heat) and C_4 (testis). *J Biol Chem* **258:** 7029–7032.

Li SS-L, O'Brien DA, Hou EW, Versola J, Rockett DL, and Eddy EM. 1989. Differential activity and synthesis of lactate dehydrogenase isozymes A (muscle), B (heart) and C (testes) in mouse spermatogenic cells. *Biol Reprod* **40:** 173–180.

Liang ZG, Shelton JA, and Goldberg E. 1986. Non-cross-reactivity of antibodies to murine LDH-C_4 with LDH-A_4 and LDH-B_4. *J Exp Zool* **240:** 377–384.

Mahi-Brown CA, VandeVoort CA, McGuinness RP, Overstreet JW, O'Hern P, and Goldberg E. 1990. Immunization of male but not female mice with the sperm specific isozyme of latate dehydrogenase (LDH-C_4) impairs fertilization *in vivo*. *Am J Reprod Immunol* **24:** 1–8.

Markert CL. 1963. Lactate dehydrogenase isozymes: dissociation and recombination of subunits. *Science* **140:** 1329–1330.

Market CL, Shaklee JB, and Whitt GS. 1975. Evolution of a gene. *Science* **189:** 102–114.

Melchers I, Rajewsky K, and Schreffier DC. 1973. Ir-LDH_B: Map position and functional analysis. *Eur J Immunol* **3:** 754–761.

Millan JL, Driscoll CE, LeVan KM, and Goldberg E. 1987. Epitopes of human testis specific lactate dehydrogenase from cDNA sequence. *Proc Natl Acad Sci (USA)* **84:** 5311–5315.

Mita M and Hall PF. 1982. Metabolism of round spermatids from rats: lactate as preferred substrate. *Biol Reprod* **26:** 445–455.

Musick WDL, Adams AD, Rossman MG, Wheat TE, and Goldberg E. 1976. A low resolution study of testicular lactate dehydrogenase using molecular replacement technique. *J Mol Biol* **104:** 659–668.

Musick WDL and Rossman MG. 1979. The structure of mouse testicular lactate dehydrogenase-C_4 at 2.9 Å resolution. *J Biol Chem* **254:** 7611–7620.

O'Hern. 1991. Immunogenicity of peptide having predetermined alpha-helical and alpha-alpha fold topologies. *Mol Immunol* **28:** 1047–1053.

O'Hern P, Bambra CS, Isahakia M, and Goldberg E. 1995. Reversible contraception in female baboons immunized with a synthetic epitope of sperm specific lactate dehydrogenase. *Biol Reprod* **52:** 331–339.

Pan Y-CE, Sharief FS, Okabe M, Huang S, and Li SS-L. 1983. Amino acid sequence studies on lactate dehydrogenase-C_4 isozymes from mouse and rat testes. *J Biol Chem* 7005–7016.

Pawlowski R and Brinkmann B. 1992. Evaluation of sperm specific lactate dehydrogenase isozyme C_4 (LDH-C_4)-application of semen detection in stains. *Int J Legal Med* **105:** 123–126.

Prasad P, Mumford D, and Gordon H. 1976. Lactate and malate dehydrogenase and α-esterases in oligospermia. *Fertil Steril* **27:** 7–14.

Reader SC, Shingles C, and Stonard MD. 1991. Actue testicular toxicity of 1, 3 dinitrobenzene and ethylene glycol monoethyl ether in the rat: evaluation of biochemical effect markers and hormonal responses. *Fundam Appl Toxicol* **16:** 61–70.

Sakai I, Sharief FS, and Li SS-L. 1987. Molecular cloning and nucleotide sequence of cDNA for sperm specific lactate dehydrogenase-C from mouse. *Biochem J* **242:** 619–622.

Salehi-Ashtiani K and Goldberg E. 1993. Differences in regulation of testis specific lactate dehydrogenase in rat and mouse occur at multiple levels. *Mol Reprod Dev* **35:** 1–7.

Sarma RH and Kaplan NO. 1970. *Proc Natl Acad Sci USA* **67:** 1375–1380.

Schatz L and Segal HL. 1969. Reduction of α-ketoglutarate b7 homogeneous lactic dehydrogenase-X of testicular tissue. *J Biol Chem* **244:** 4393–4397.

Shelton JA and Goldberg E. 1985a. Induction of cell-mediated cytotoxic immunity to sperm-specific lactate dehydrogenase-C_4 in SJL/J female mice. *Biol Reprod* **32:** 556–560.

Shelton JA and Goldberg E. 1985b. Serum antibodies to LDH-C_4. *J Reprod Immunol* **8:** 321–327.

Shelton JA and Goldberg E. 1986. Local reproductive tract immunity to sperm specific lactate dehydrogenase-C_4. *Biol Reprod* **35:** 873–876.

Short S, Short ML, Milkowski DM, and Jungmann RA. 1991. Functional analysis of *cis*- and *trans*-regulatory elements of lactate dehydrogenase A sub-unit promoter by *in vitro* transcription. *J Biol Chem* **266:** 22164–22172.

Szeinberg A, Mor A, Verma H, and Reischer S. 1966. "Band-X" isozyme of lactic dehydrogenase in pathological spermatogenesis. *Life Sci* **5:** 1233–1235.

Takano T and Li SS-L. 1989. Human testicular lactate dehydrogenase-C gene interrupted by six introns at positions homologus to those of Ldh-a (muscle) and Ldh-b (heart) genes. *Biochem Biophys Res Commune* **159:** 579–583.

Takatera H, Nomura F, and Itatani H. 1993. Differential expression of human sperm specific isozyme in seminoma cells transplanted in scid-nude mice. *Jpn J Cancer Res* **84:** 215.

Taylor SS and Oxley SS. 1976. Homolgoies in the active site regions of lactate dehydrogenases. *Arch Biochem Biophys* **175:** 373–383.

Thomas K, Del Mazo J, Eversole P, Bellve A, Hiraoka Y, Li SS-L, and Simon M. 1990. Developmental regulation of expression of the lactate dehydrogenase (LDH) multigene family during mouse spermatogenesis. *Development* **109:** 483–493.

Velasco NJA, Tover-Zepata I, Martinez-Hernandez P, Perez-Allbacete M, Tortosa-Oltra J, and Parrilla-Parico JJ. 1993. Lactic dehydrogenase-C_4 activity in seminal plasma and male infertility. *Fertil Steril* **60:** 331–335.

Virji N and Eliasson R. 1985. LDH-C_4 in human seminal plasma and testicular function. IV. Relationship to other semen variables. *Int J Androl* **8:** 376–384.

Wang SX, Luo AM, Liang ZG, Song JF, Wang HA, and Chen YX. 1990. Preparation and characterization of monoclonal antibodies against sperm-specific lactate dehydrogenase C_4. *J Androl* **11:** 319–324.

Warren WA. 1970. Stereochemistry of glyoxylate oxidation by NAD and mammalian lactate dehydrogenase. *Biochim Biophys Acta* **212:** 503–510.

Wheat T and Goldberg E. 1975. LDH-X: the sperm-specific C_4 isozyme of lactate dehydrogenase. In: *Isozymes III. Development Biology.* Markert CL, Ed. New York, Academic Press, pp. 325–346.

Wheat TE and Goldberg E. 1976. Induction of infertility in male rabbits by immunization with LDH-X. In: *Regulatory Mechanisms of Male Reproductive Physiology.* Spilman et al., Eds. Excerpta Medica, Amsterdam, pp. 133–137.

Wheat TE and Goldberg E. 1983. Sperm-specific lactate dehydrogenase C_4: antigenic structure and Immunosuppression of fertility. In: *Isozymes: Current Topics in Biological and Medical Research.* Vol. 7, Rattazzi MC,

Scandalios JG, and Whitt GS, Eds. New York, Alan R. Liss, pp. 113–130.

Wheat TE, Goldberg E, and Margoliash E. 1977. Primary structure of the essential thiol peptide from lactate dehydrogenase-c subunit. *Biochem Biophys Res Commun* **74:** 1066–1070.

Wheat TE and Goldberg E. 1985a. Antigenic domains of the sperm-specific lactate dehydrogenase C_4 isozyme. *Mol Immunol* **22:** 643–649.

Wheat TE and Goldberg E. 1985b. Immunochemical dissection of testis specific lactate dehydrogenase C_4. *Ann NY Acad Sci* **438:** 156–169.

Wheat TE, Shelton JE, Gonzales-Prevatt VB, and Goldberg E. 1985. The antigenicity of synthetic peptide fragments of LDH-C_4. *Mol Immunol* **22:** 1195–1199.

Whitt G. 1984. Genetic developmental and evolutionary aspects of the lactate dehydrogenase isozyme system. *Cell Biochem Func* **2:** 134–139.

Wong C, Rodriguez-Paez L, Nogueda B, Perez A, and Baeza I. 1997. Selective inhibition of the sperm specific lactate dehydrogenase isozyme-C_4 by N-isopropyl oxamate. *Biochim Biophys Acta* **1343:** 16–22.

Wright LL and Swafford JH. 1984. Mouse lactate dehydrogenase (LDH) C_4 (Testis) is immunochemically cross-reactive with LDH-A_4 (muscle) and LDH-B_4 (Heart). *Scand J Immunol* **19:** 247–254.

Wu KC, Chan K, Lee CYG, and Lau YF. 1987. Molecular isolation and sequence determination of the cDNA for the mouse sperm specific lactate dehydrogenase-X gene. *Biochem Biophys Res Commun* **146:** 964–970.

Yoshida K, Ochijima Y, and Saito H. 1989. A further study of human seminal plasma lactate dehydrogenase-C_4 (LDH-C_4). *Hinyokika Kiyo* **35:** 457–463.

Zhou W, Xu J, and Goldberg E. 1994. A 60 bp core promoter sequence of murine lactate dehydrogenase-C is sufficient to direct testis-specific transcription *in vitro*. *Biol Reprod* **51:** 425–432.

Zhou W and Goldberg E. 1996. A dual function palindromic sequence regulates testis-specific transcription of the mouse lactate dehydrogenase C gene *in vitro*. *Biol Reprod* **54:** 84–90.

Zinkham WH, Blanco A, and Clowry L. 1964. An unusual isozyme of lactate dehydrogenase in mature testes: Localization, ontogeny and kinetic properties. *Ann NY Acad Sci* **121:** 571–588.