BLADDER RECONSTRUCTION WITH AMNIOCHORION

By

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B.Sc., Simon Fraser University, 1979

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

In
THE FACULTY OF GRADUATE STUDIES
(DEPARTMENT OF PATHOLOGY)

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THE UNIVERSITY OF BRITISH COLUMBIA
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By Susan Lorraine Ettinger

Bladder reconstruction has been used since 1851 to surgically enlarge small capacity bladders but various materials grafted onto the bladder remnant, have proved inadequate because of leaky anastomoses, fibrous contracture and encrustation or stone formation. Such inadequacies have continued to prompt investigators to seek alternatives.

In the present studies, supratrigonal cystectomies were performed in rabbits and then bladder reconstructions were attempted using one, two or four layers of human amniochorion. Control rabbits underwent supratrigonal cystectomy then closure either with or without the detached bladder remnant. It was hypothesized that amniochorion because of its tensile strength and slow rate of degradation may function as a scaffold for regenerating bladder tissue while maintaining bladder size and function. The abilities of amniochorion: a) to restore bladder size and function was assessed by measuring bladder capacities before and after surgery; and b) to serve as a temporary scaffold was assessed by the immunologic response and graft degradation, and by the extent of bladder wall regeneration.

Bladder capacities were significantly reduced when measured post-operatively, at 4 weeks or less, in both control and experimental groups and this may have been caused by surgical trauma, inflammation and irritation. However, bladder capacities measured from 5 to 17 weeks post-operatively appeared to increase with time, which may have been the result of decreased inflammation and increased quantities of smooth muscle within regenerating bladder wall.

Human amniochorion provoked an immune response when grafted into the rabbit bladder as cytotoxic antihuman antibodies were detected in rabbit sera in a complement-dependent cytotoxicity (CDC) assay using human lymphocytes. Platelet-absorbed sera in some cases produced a negative CDC response reflecting elimination of HLA antibodies and in other cases
produced a positive response perhaps indicating the presence of antibodies to a lymphocyte/trophoblast crossreacting antigen. Platelet-absorbed sera in an indirect immunofluorescent assay, using amniochorion, produced positive fluorescence suggesting the presence of antibodies to amniochorion-associated antigens. Absorption with amniochorion, however, removed this reactivity which suggests that some of the antibodies were specific for amniochorion. Amniochorion-absorbed sera produced a positive CDC assay indicating the presence of antibodies cytotoxic to human lymphocytes (perhaps due to HLA antigens). Further evidence of an immune reaction, was the immediate assemblage of inflammatory cells (lymphocytes, polymorphonuclear cells, mast cells and macrophages) and their persistence for several weeks. Remnants of amniochorion could be identified after 9 weeks. Stone formation, probably due to necrotic debris, occurred in 44% of rabbits with single-layered and 87-90% with multi-layered grafts.

Histological studies showed that granulation tissue bridged the defect during the first 3-4 weeks, and that urothelium, apparent at 5 weeks, attained normal thickness after 9 - 16 weeks. Few smooth muscle cells extended into the defect area as early as 5 weeks but after 11-17 weeks were numerous and organized into bundles. However, collagen usually comprised the greatest proportion of tissue in the regenerated wall.

This study showed that human amniochorion, while maintaining continence and bladder function, acted as a temporary scaffold for connective tissue overgrowth and urothelial and smooth muscle regeneration. Despite an intense chronic inflammatory response and abundant collagen, a result of ischaemic necrosis and rejection, complete contracture did not occur possibly because of adequate smooth muscle regeneration. Studies using amniochorion as an allograft are required to determine if fibrous contracture occurs after several months and whether or not stone formation can be avoided with diuretic therapy. Amniochorion may become an alternative material to the less than adequate bowel graft currently used.
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ACKNOWLEDGEMENTS

Janet Sadoway, Teri Dahlgren, and Brenda Draney of the Immunology Laboratory at Vancouver General Hospital are to be commended for their excellent technical assistance and sound advice. Their contribution to this study is greatly appreciated.

Eva Germann, Programmer Analyst of the Computer Programming Services of the Department of Surgery, assisted with the statistical analysis of our data and I thank her for her helpful suggestions and ideas.

I am grateful to John Smith-Weston and Ron Thorpe of the Department of Biomedical Communications for their excellent work.

Fred Jensen of the Animal Care Facility at CCABC deserves special mention for the wonderful care he provided our animals throughout the study.

It was a great honor and privilege to have Dr. John Brown, Dr. Jorge Denegri, Dr. Bill Godolphin, Dr. Jim Hudson, Dr. Noel Quenville and Dr. Martin McLoughlin on my supervisory committee. I would like to thank them all for their dedication to seeing this study to its successful completion and for their thoughtfulness. I thank Dr. Brown for reviewing the thesis and for his constructive suggestions and encouragement. I thank Dr. Denegri for introducing me to the fascinating field of Immunology, for his time, direction, support and encouragement. I thank Dr. Godolphin for providing direction and for his many hours and valuable advice. Dr. Quenville reviewed numerous histological slides and his suggestions and kindness are greatly appreciated. I would like to thank Dr. McLoughlin for his enthusiastic support and encouragement, and for his kindness.

I am grateful to the examiners, Dr. Doug Kilburn and Dr. Paul Keown for their thought-provoking questions and their consideration.

My family, Harry and Doreen Pride, Ken and Marilyn Pride, Kathleen and Gary Harper and Sharon and Willie Postler deserve special mention for their kindness and support.

This thesis is dedicated to my wonderful husband Kim Lorne Ettinger whose understanding, support and encouragement enabled me to continue my studies and successfully complete this degree.
INTRODUCTION

A small bladder capacity results in an increased rate of micturition and when patients are forced to void every 15 - 30 minutes the bladder ceases to function as a low pressure reservoir with incontinence (loss of bladder control) as the final outcome. A decreased capacity can result from inflammatory conditions such as interstitial cystitis and tuberculous cystitis which cause scarring and contracture of the bladder or it can also be a manifestation of multiple sclerosis or spastic neuropathic bladders in which nervous signals controlling bladder contraction are uncoordinated. Inadequate quantities of bladder tissue following tumour removal, pelvic exenteration for tumour, trauma, posterior urethral valves and congenital malformation of the abdominal wall and anterior bladder wall (exstrophy) also necessitate reconstruction.

Treatment of the small-volume bladder consists of anticholinergic drugs and dilation programmes for diseased bladders and when these forms of management are ineffective or when the capacity is decreased for structural reasons, surgical reconstruction is necessary. The first bladder reconstruction to successfully provide a functional capacity was reported by Tizzoni and Foggi in 1888 in which an isolated segment of ileum was attached to the bladder neck in a dog to increase vesical capacity. This form of reconstruction was not attempted clinically until 1899 when Mikulicz anastomosed a loop of ileum to the bladder in a patient with tuberculous cystitis. Over the next 89 years numerous studies of bladder reconstruction using biologic and nonbiologic grafts were undertaken, in which the grafts were tested for their effectiveness as bladder wall replacements and for their ability to maintain an adequate capacity. Intestinal grafts included segments from the stomach, ileum, cecum, colon and rectum but complications such as incomplete emptying, infections, stones, mucus secretion and electrolyte abnormalities were encountered and prompted investigators to use non-intestinal grafts. Non-intestinal biologic grafts such as lyophilized human dura, fascia, omentum, peritoneum, pericardium, bladder mucosa and amniochorionic membranes were tested but most grafts became scarred and contracted, resulting in small capacity bladders.
Artificial materials were also incorporated into human and animal bladders during experimental reconstructive surgeries in which plastic moulds, and grafts made of silastic, polyvinyl sponge, teflon felt, nylon-velour-silastic and synthetic alpha-amino acid membranes were used. These grafts were frequently extruded into the bladder lumen, bladder wall regeneration was non-existent in most cases and functional capacities were usually inadequate.

While most biologic grafts proved inadequate for bladder reconstruction, human amniochorion has been used somewhat successfully in rabbits and dogs. The capacities observed radiographically after 4 weeks were reported to be adequate and the bladder wall showed some evidence of regeneration. These results were remarkable considering the possible immunologic sequelae such as chronic inflammation in response to the xenograft with subsequent scarring of the bladder wall. To effectively reconstitute the bladder, such that the capacity remains functional, smooth muscle as well as urothelium must regenerate and scarring must not be extensive.

The present study was designed to assess the effectiveness of amniochorion as both a bladder wall substitute to maintain or increase bladder capacity and as a temporary scaffold for regenerating cells. To this end, the study attempted to examine bladder capacities at various post-operative intervals; whether or not an immunologic response was generated; regenerative capabilities of urothelial and smooth muscle cells; and the extent of graft degradation. This xenograft model was used despite the possible immunological response because the purpose was to test a material which provided a temporary scaffold only, as permanent grafts within the bladder wall fail to allow regeneration of bladder wall components. The immunologic reaction of amniochorion in human bladders may be less significant. Construction of a leak-proof bladder required a multiple-layer graft so 2, and 4 layered grafts were tested in addition to the single-layered graft.

The results were somewhat surprising when compared to similar experiments reported by
other investigators. The bladder capacities were not maintained following reconstruction, rather they were decreased during the first 4 weeks and then gradually increased, during the 17 week followup. Some other important findings were observed: 1) there appeared to be an acute immune response to the human amniochorion; 2) the rabbits did generate antibodies to human antigens associated with the amniochorion; 3) the xenograft did survive long enough to provide a scaffold for connective tissue regeneration along the superficial surface of the bladder; 4) urothelium regenerated after 5 weeks while scant smooth muscle cells appeared at the margins to infiltrate the defect; 5) after several weeks collagen was still abundant within the regenerated wall but smooth muscle cells now formed small bundles and 6) there was crystalline encrustation of the luminal layer of the graft with subsequent stone formation.
Part One: REVIEW of the LITERATURE
I. Evolution of the Reconstructed Bladder

Treatment for small capacity or diseased bladders was almost non-existent during the first half of the 19th century, then over the next one hundred years numerous surgical techniques were devised including: urinary diversion to the intestinal tract or to an isolated segment of intestine; bladder reconstruction with tissue or synthetic grafts; and bladder replacement with prosthetic grafts. The early techniques were fraught with complications, many of which proved to be fatal and while the more recent developments overcame some of the earlier problems, bladder reconstruction today remains a major surgical problem.

1 Urinary diversion to intact intestine or isolated segments of intestine

Ureters were attached to the ileum, cecum, colon, rectum or stomach in an effort to divert the urine to the gastrointestinal tract so that the bowel served as a reservoir for both urine and faeces. Complications were encountered though and were usually associated with inadequate ureteral anastomoses to the bowel: urinary leakage at the site of anastomosis resulted in peritonitis; ureters were often obstructed because of edema and stricture; and reflux of intestinal content to the upper urinary tract caused pyelonephrosis and renal deterioration. Attachment of the ureters to the bowel was one of the greatest challenges with this form of bladder reconstruction.

Numerous techniques were used to implant the ureters into the intestinal segment as documented by Hinman and Weyrauch, and Thorne and Resnick, who reviewed the major advances in uretero-intestinal anastomosis occurring between 1851 and 1968. Three techniques formed the basis for the anastomoses used today: mucosa-to-mucosa attachment; ureteral tunneling through intestinal submucosa; and construction of an antirefluxing nipple.
Despite the increased incidence of patient survival with the new reconstructive procedures, reports of chronic complications began appearing in the literature in the late 1950’s and 1960’s. Chronic metabolic acidosis, potassium deficiency, an increased incidence of colonic cancer, bowel frequency and incontinence led to a decreased usage of intestine for urinary diversion. 26-29.

Another technique used during the first half of this century involved isolating the intestinal bladder from the rest of the bowel. Rubin4 reviewed over twenty cases in the French and German literature and reported such uses of intestinal urinary reservoirs or conduits which were emptied either through the anal sphincter, a perineal stoma, or a perineal urethra. From this review it became apparent that the problems of ascending infection with subsequent destruction of renal tissue had not been eliminated.

An alternative location for the stoma, designed to remove the urinary stream from the faecal stream, was the abdominal wall. Verhoogen30 attempted to create a urinary reservoir from an ileocecal segment then used the appendix to divert the urine to an abdominal stoma. Gallo31 implanted the ureters into an isolated caecal pouch and discovered 13 years later that the patient’s urinary tract was still normal. Gilchrist32 created an isolated ileocaecal urinary bladder, in a patient with bladder cancer, in which the ileal limb was brought to an abdominal wall stoma and intermittent catheterization was used to empty the bladder. Cordonnier and Lage33 extended this technique and used an isolated caecal or rectal pouch to form a urinary reservoir, to overcome complications of kidney infection related to the inadequate uretero-intestinal anastomosis with the ileum.

Further experimental and clinical experiences with reconstructed bladders made from intestinal segments were reported by Bricker34, Merricks et al35 and Glaser36 during the 1950’s. Caecum and ascending colon were formed into a reservoir which communicated with the skin by an ileal conduit. The bladders were not continent, patients had to rely on indwelling
catheters or collection bags and they developed moderate hydronephrosis. Bricker\textsuperscript{34} also used
segments of sigmoid or ileum and caecum as conduits through which urine drained into a
collection device on the abdomen. Complications relating to infection, calculus formation,
reflux and renal deterioration, and stomal ulceration contributed to such a poor quality of life that
thirty percent of the patients withdrew from normal daily activities.\textsuperscript{37} Another reconstruction
technique which had been investigated as early as 1888 involved anastomosing an isolated
segment of bowel to the bladder base in an attempt to preserve the normal route of voiding.

2 Isolated Segments of Bowel Anastomosed to the Bladder Remnant

Since Tizzoni and Foggi\textsuperscript{1} first reported their experiences with ileum to increase bladder
capacity in a dog various isolated intestinal segments, from the ileum to the rectum, have been
used to reconstruct bladders. The preferred choice of bowel segment changed as problems were
encountered and solved and initially ileum and rectum were used. Mikulicz\textsuperscript{2} was the first to
apply Tizzoni and Foggi's operation to humans when he used a segment of ileum to augment a
patient's bladder capacity and was followed by Rutkowski\textsuperscript{38} who attached an ileal patch to his
patient's bladder to increase its capacity. The next clinical account of anastomosing an "artificial
bladder" to a bladder remnant or to the urethra was reported by Lemoine.\textsuperscript{39} He implanted the
ureters into the rectum as others had, but then because his patient suffered from ascending
infection, he isolated the rectum and formed a urinary reservoir. The rectal bladder was sutured
to the urethra then the sigmoid was sutured to the anus and was thus under anal sphincteric
control. While this procedure was practical and formed the basis of a similar procedure used
today the patient died of sepsis on the eighteenth post-operative day. A different design in which
a ring of small intestine was anastomosed to the bladder itself appeared successful but despite
two cases of normal urination the pre-operative symptoms of urgency and frequency were not
eliminated.\textsuperscript{40}

During the 1940's experiences using sigmoid to reconstruct the bladder began appearing in
the literature. Rubin\textsuperscript{4} attached the sigmoid to the urethra in a number of dogs and found that 7
of the 11 dogs were continent and voided normally and despite the fact that there was residual urine, ascending renal infection was not evident. Bisgard\textsuperscript{41} also experimenting on dogs, anastomosed a segment of sigmoid to the urethra and reported that after 4 months the upper urinary tract had not deteriorated and there was no hydronephrosis. Later Bisgard and Kerr\textsuperscript{42} anastomosed the distal sigmoid to the urethra in dogs and despite the small capacities attained, ranging from 15 - 35 cc, the animals appeared continent and there were no cases of upper urinary tract infection or hydronephrosis.

During the fifties and sixties controversies developed over which intestinal segment was the most suitable for bladder reconstruction. Couvelaire\textsuperscript{43} first reported bladder reconstruction using caecum and stressed the importance of leaving the trigone intact to preserve sensory function. Twelve years later Gil-Vernet et al\textsuperscript{44} discussed the results of 41 patients who had undergone reconstruction with sigmoid and colon. Fistulas at the urethrosigmoid anastomosis occurred in 12 patients, 2 patients died from paralytic ileus, and 1 of 4 patients with ureterohydronephrosis died of renal destruction. Ureteral reflux and ascending pyelonephritic infection were noted in almost all cases but despite the numerous complications there were some positive results as well: mucus formation gradually disappeared, the new bladder distended with time so that after 1 - 2 years patients were voiding every 3 - 5 hours and residual urine was usually less than 40 ml. At night or during sound sleep, however, only 3 patients had good urinary control. So, both good and poor results were obtained when bladders were constructed with caecum, sigmoid or colon.

Ferris\textsuperscript{45} reported that his patients with ileal reconstruction of the bladder were able to empty their new bladders completely. Capacities ranged from 300 to 400 ml and pre-operative symptoms of frequency, urgency and pain disappeared. Then Hradec\textsuperscript{46}, operating on 114 patients between 1957 and 1963, substituted all or part of the bladder using various intestinal grafts; the ileal segment; the sigmoid segment and the ileocaecal segment. He concluded that expulsion of urine from the intestinal bladder was best with caecum or sigmoid because voiding
velocities were comparable to those of normal bladder and patients with ileal bladders were incontinent or had nocturnal enuresis. Capacities of the artificial bladders ranged between 200-400 ml, there was a 10% occurrence of calculi and reflux was a common finding. Cibert\textsuperscript{47} reported on 18 cases of ileocystoplasty for bladder augmentation in which 4 patients died from the operation and 12 of the remaining 14 had improved bladder function. That same year Tasker\textsuperscript{48} used a patch of ileum to augment the bladder because he found that using a loop of intestine led to stagnation of urine within the loop due to inefficient voiding and a patch also reduced the possibility of narrowing at the anastomosis.

The ileocolic segment was used to reconstruct the bladder and provided the patients with good capacities, normal voiding intervals and forceful urinary streams.\textsuperscript{49} Protection provided by the ileocaecal valve diminished or prevented reflux to the kidney. Gil-Vernet\textsuperscript{49} chose to use the sigmoid colon in 158 cases of bladder reconstruction because of the storage capacity it provided and the voiding velocity it generated. The ileum was used less extensively because the capacity was less and the voiding pressures lower. Kuss\textsuperscript{50} preferred to use the pelvic colon after 3 of 5 patients suffered intestinal occlusion (volvulous, adhesions) when ileum was used to reconstruct the bladder. Between 1957-59 he used the colon in 19 patients and reported 1 case of obstructed intestines due to an abscess, 4 cases of urinary fistulas, and 1 colic fistula. The quality of micturition was superior though because the contractile forces enabled a greater voiding velocity than was possible with other segments of bowel.

Carney and Leduc\textsuperscript{51} and Lilien and Camey\textsuperscript{52} introduced an entirely different procedure in which a U-shaped segment of ileum or colon was anastomosed to the urethra and the ureters were implanted into each end in an anti-refluxing manner. There was an initial mild hyperchloremic acidosis which eventually stabilized and while ninety percent of the patients were continent during the day most patients experienced night-time frequency with some patients requiring a collection device.
During the 1970's and '80's, it became apparent that regardless of the segment of bowel used, the peristaltic contractions were all equally inefficient at expelling urine compared to normal bladder musculature, the extent of electrolyte reabsorption did not differ significantly between the ileum and colon and the amount of mucus secreted was similar. The ileal, ileocaecal, caecal and sigmoidal segments were all considered useful. Smith et al\textsuperscript{53} reported augmentation cystoplasty in 74 patients in which there were 45 cases of ileocystoplasty, 16 of ileocaecocystoplasty, 7 sigmoidocystoplasty and 6 caecocystoplasty. Twenty patients had excellent results, 23 had good results and there were 26 failures but the bowel segment used did not influence the outcome. Complications included an imbalance of micturition resulting in either urinary retention or incontinence and while some patients with pre-operative reflux had improvement post-operatively, 8 patients without pre-operative reflux developed reflux after the reconstruction procedure. Two patients developed progressive uraemia and technical complications included 1 fibrotic intestinal patch due to ischaemia, 1 contracted bladder-intestinal anastomosis and 5 bladders with hourglass configuration with possible residual urine.

Chan et al\textsuperscript{54}, documented good results in 17 patients with caecocystoplasties with only 1 technical failure and 1 recurring urinary tract infection. Dounis et al\textsuperscript{55} reported their experiences with caecocystoplasty in 37 patients over 15 years. Urinary tract infections persisted in 10 patients, abnormal micturition was present in 11 patients, bladder outflow obstruction occurred in 5 patients and 6 suffered from urinary fistulas. Capacities ranged from 200 - 700 ml and voiding was achieved by abdominal straining. Skinner\textsuperscript{56} performed ileocaecocystoplasties in 23 patients and reported that two patients had reflux across the ileocaecal valve resulting in recurrent pyelonephritis, 1 patient remained incontinent after 18 months, 1 patient had nocturnal enuresis but all other patients had good results. Koskela and Kontturi\textsuperscript{57} performed enterocystoplasties in 15 patients and demonstrated that good or satisfactory voiding was achieved in 14 cases. Sigmoidal bladders emptied the most efficiently because sensations of fullness provided the patients with a desire to void. Contraction of the sigmoid and abdominal straining allowed the patients to empty their new bladders and while the caecal bladders emptied
satisfactorily the sensation to void was weaker than that with the sigmoid. Good micturition occurred in 2 patients with ileal bladders but there were problems of stress incontinence and nocturnal enuresis. All 4 patients with ureteroileocystoplasties had good micturition. Fall and Nilsson\(^5\) reported that of 12 patients with sigmoid or caecocystoplasties, results were good in 7, poor in 5 and most patients suffered from nocturia once or twice nightly.

Capacities achieved with these enterocystoplasties were adequate but incontinence, reflux and chronic emptying problems were common complications. However, as timed intermittent catheterization gained approval many of these complications were overcome. Whitmore and Gittes\(^5\), during a 15 year period, reconstructed bladders in 45 patients. Thirty-two were considered successes as the reconstruction remained intact and renal function was stabilized. The failures, which included patients with progressive renal deterioration, electrolyte abnormalities, and no relief from pre-operative symptoms, required twenty additional operations to achieve success. Reflux was a problem in 6 patients who developed it subsequent to chronic emptying problems and 6 patients had hyperchloraemic acidosis which was controlled by oral bicarbonate. No patients were able to sleep through the night without getting up to void and bed-wetting occurred in 1/3 to 1/2 of the patients because of a decreased perception of fullness. This procedure did offer an improved capacity and urinary continence (with timed voiding and intermittent catheterization) and most patients had relief from pre-operative symptoms. Kass and Koss\(^6\) reported good results in 13 out of 14 patients provided intermittent catheterization was performed every 4 hours. Linder et al\(^6\) were able to help 14 out of 18 patients achieve continence by performing caecocystoplasty and ileocystoplasty and again the segment of bowel used did not appear to affect the results. Conversely Lockhart et al\(^6\) concluded after their results in 15 patients that the small bowel stored larger amounts of urine at a lower detrusor pressure than the large bowel. Those with ileocystoplasty were continent with intermittent catheterization while those with caecosigmoidocystoplasty required intermittent catheterization and anticholinergic drugs to maintain continence. Thuroff et al\(^6\) attached the continent ileal Kock pouch, in which a urinary reservoir is constructed from ileum, to the membranous urethra.
Ten of eleven patients were dry day and night with normal voiding intervals and while most voided spontaneously and effectively with little residual urine 2 patients required intermittent catheterization to empty their bladders. These impressive results have since encouraged others to perform such operations and while patients generally suffer from fewer complications, the antirefluxing valves have required some further revisions.

In summary, techniques of bladder reconstruction which maintained the normal route of voiding resulted in fewer complications than techniques using urinary diversion. There was less incidence of reflux and renal deterioration, and bladder capacities were improved. However, a technique without complications was still being sought as paralytic ileus was occasionally fatal, pre-operative symptoms persisted in some patients, mucus formation occurred continually, and incontinence, and inefficient voiding compromised the patient’s lifestyle.

While some investigators were reconstructing bladders with segments of intestine, others were studying the effectiveness of tissue patch-grafts to replace various portions of the bladder wall.

3 Bladder Reconstruction With Viable Tissue Grafts

Intestinal as well as nonintestinal tissue-grafts were used to reconstruct bladders. Intestinal grafts included ileum and colon and nonintestinal grafts included fascia, omentum, peritoneum, pericardium, bladder mucosa, and amniotic membranes.

Interaction between urine and intestinal mucosa had been a problem with intestinal substitution of the bladder because the mucosa retained its inherent properties of absorbing electrolytes and nitrogenous wastes, and of producing mucus. In an attempt to eliminate some of these problems, Grotzinger et al64 and Shoemaker et al11 anastomosed ileal or colonic grafts, stripped of their mucosal layers, to the bladder trigone with the serosal surface positioned towards the bladder lumen. Transitional epithelium had regenerated after 2-3 weeks and after 6 months intestinal musculature had apparently re-organized to resemble detrusor muscle. Bladder
capacities returned to normal and there were no incidences of mucus formation nor electrolyte
abnormalities. This technique was first attempted in a patient in 1954 and during the first year
the patient suffered from a persistent proteus infection and he developed calculi, which required
removal, but the capacity was adequate and the urine was clear. The experience of Campbell with this technique was less than satisfactory as the graft contracted and atrophied in his 2 patients. This method of reconstructing an artificial bladder did not gain popularity.

Bladders were also repaired with non-intestinal grafts such as fascia, peritoneum, omentum
and pericardium. Hohmeier used fascial grafts to bridge bladder defects but his results were
generally poor as the graft became perforated causing fatal peritonitis. Neuhof also
transplanted fascial grafts into partially resected bladders but described his results as successful
because urothelium and smooth muscle did regenerate. However, osteoid plaques, cartilage,
bone marrow and periosteum developed at the transplant site. Huggins reported similar results
when fascia was used to repair the bladder in dogs as lamellar membranous bone developed
within 3 or 4 weeks post-operatively. During the 1950's Baret and DeMuth et al used fascia
to repair experimentally induced bladder extrophy (where the internal surface of the bladder
communicated with an opening in the abdominal wall), but the fascial grafts had contracted by
more than 50% and osteoid plaques developed. Peritoneum was used more recently by Jelly and while he found that the peritoneal graft acted as a scaffold for regenerating urothelium, gradual fibrosis of the graft occurred. In 1966 Goldstein and Dearden, successfully
reconstructed rabbit bladders with segments of omentum as the urothelium and smooth muscle
appeared to regenerate from the bladder margins.
Novick\textsuperscript{69}, using a rabbit model, was able to construct an artificial bladder from bovine pericardium which provided an adequate capacity. After 6 weeks urothelial regeneration was complete and after 14 weeks smooth muscle bundles were present throughout the bladder but the presence of microfoci of bone discouraged further investigations with pericardium.

Fresh human amniotic membranes were used to augment partially resected rabbit bladders. Norris et al\textsuperscript{20} noted some early post-operative leakage and calculi on suture remnants, but urothelial regeneration was evident by the end of the fourth week. The bladder walls were inflamed and amniotic membranes were reduced to microscopic foci, the stroma was scarred and no smooth muscle bundles were apparent after 4 weeks. Fishman et al\textsuperscript{146} and Flores et al\textsuperscript{70} reported similar experiences with amniotic membrane grafts in rabbit bladders.

Of the viable tissue-grafts used to reconstruct bladder, omentum and amniochorion presented the fewest problems and adequate capacities were apparently achieved. The use of nonliving tissue-grafts to repair bladder defects was also being investigated.

4 Bladder Reconstruction With Preserved-tissue Grafts

Preserved tissue grafts such as fascia, bladder, human dura and amniotic membranes were used experimentally to repair bladders. Koontz\textsuperscript{71} replaced the bladder dome in dogs with alcohol-preserved ox fascia lata and he noted that fibrin spread over the implant and that the urothelium was completely regenerated by 3 months. Capacities were not mentioned, nor was the observation of smooth muscle regeneration so a longer follow-up might have revealed a scarred and contracted graft.

Bladder grafts preserved in alcohol formalin were anastomosed to partially resected bladders by Tsuji\textsuperscript{12} and while the grafts were covered with proliferating connective tissue on the serosal surface they gradually became detached and were finally discarded into the bladder lumen.
Smooth muscle was apparent only adjacent to the edges of the bladder remnant and did not extend into the reconstructed area. The size of the bladder was reduced but the original bladder gradually enlarged to provide what the authors termed "an adequate capacity", although no measurements were provided.

Lyophilized human dura was used experimentally and clinically by Kelami et al in 1970 to augment resected bladders. Initial results appeared favourable because urothelium regenerated and the dura was eventually resorbed. Others, such as Hansen, reported less satisfactory results when lyophilized dura was implanted into the bladders of dogs. Three dogs died of urinary leakage, the dura graft was gradually replaced by scar tissue which contracted to produce a small-capacity bladder and although epithelial regeneration was observed there was no regeneration of smooth muscle cells and subepithelial bone formation was noted after 3-4 months. When Kelami reported long-term results in 1975, there was still no evidence of muscle regeneration and 1 patient suffered from a perforated graft which eventually led to a vesico-intestinal fistula and peritonitis.

Amniotic membranes preserved in glutaraldehyde then tested as grafts in rabbit bladders resulted in early leakage which was rectified by a different suturing technique. Urothelium had regenerated by 4 weeks but smooth muscle had not.

Bladder reconstruction with preserved tissue grafts was generally unsuccessful as grafts were either extruded into the lumen of the bladder or were retained but were replaced by scar tissue which eventually contracted to produce a small capacity bladder.

5 Bladder Reconstruction With Synthetic Materials

 Numerous synthetic materials have been used over the last thirty years but have met with limited success. Sanchez et al replaced a cancerous bladder with a plastic mould into which the ureters were fixed and reported that after 3 months a new bladder had developed. Connective tissue regenerated over the mould and was followed by urothelial regeneration from
the vesical neck so that the resulting bladder wall thickness was over 1 cm. Smooth muscle ingrowth was not noted and there were calcareous deposits inside and outside the mould. The patient needed to void every 3 hours and experienced mild incontinence. Bohne also used a plastic mould and achieved similar results but he did report some smooth muscle ingrowth.75

Polyvinyl sponge grafts used to reconstruct dog bladders failed to form a fibrous union with bladder tissue and were extruded into the lumen. There was microscopic evidence of a foreign body reaction and calcium salts deposited on the polyvinyl sponge.15 Ashkar et al (1968)16 reported that silastic grafts in infected dog bladders were also extruded into the lumen and were encrusted with calcium salts but that similar grafts in uninfected bladders remained intact and did not encrust. There was no urothelial regeneration in either situation. These investigators were unable to eradicate urinary infection in the presence of a permanent bladder implant. Dressler and Many76, tested a nylon-velour-silastic bladder graft in dogs. Velour enabled a water-tight anastomosis with the bladder margins but histological examination revealed scar tissue at the margins and minimal urothelial regeneration with cells extending only 1-3 mm into the velour.

The use of gelatin sponge as a bladder graft was reported by two groups, each with contrasting results. Orikasa77 and Tsuji et al78 from 1967 to 1970 used gelatin sponge as a bladder substitute in rabbits, dogs and humans and were able to increase bladder capacity. Hansen et al17 anastomosed gelatin sponge to bladder margins after partial cystectomy in rabbits but the regenerated bladder dome in all cases contracted and calculi were observed. These problems were not reported by Orikasa or Tsuji. Both groups reported urothelial and muscular regeneration but gelatin sponge as a bladder wall substitute was not pursued further.

In 1983 Koiso et al19 presented their experiences with synthetic poly alpha-amino acid membrane to replace excised bladder wall. After 6 months the membranes had been completely resorbed and the bladder wall had regenerated (urothelium and smooth muscle). There was no urolithiasis and the bladder capacities were apparently within normal limits however the authors did not describe the method used to measure capacity.
Bladders were not only reconstructed with synthetic grafts but were also completely replaced by prosthetic devices. In 1976 Auvert tested an ovoid-shaped bladder made of silicone rubber in 8 dogs. The artificial bladder was anastomosed to the bladder neck and ureters were implanted into special tubes. The only result reported was that of leakage around the bladder neck. This prosthesis was further developed by Abbou et al who provided an artificial urethra and sphincter. Three of 10 dogs had urinary leakage and in the remaining dogs residual urine was a problem, hydronephrosis was present in 4, and 5 dogs had urinary tract infections. There was no encrustation of the prosthesis, however fine gravel was present in the urine. In 1978 Kline et al developed a prosthetic bladder using urethane with a hydrogel coating. The only result reported was that the bladder emptied completely during the 8-9 day follow-up period. Further experiments are required before prosthetic grafts can be considered as an alternative method of bladder reconstruction. Inadequate function and failure to achieve leak-proof anastomoses appear to be the major concerns.

Of all the biologic and synthetic materials discussed, amniotic membranes and synthetic alpha amino acid membranes appeared to meet the criteria of a temporary bladder wall substitute as continence was maintained, the bladder wall eventually regenerated, and the graft was slowly resorbed. Long term results however were not available regarding the extent of regeneration and the eventual capacity.
II Amniochorion as a Tissue Graft

1 Development of the amnion and the chorion

The amnion and chorion are derived from trophoblast, the ectodermal tissue on the outside of the blastocyst.81,82 The amnion begins to form 7-8 days after fertilization during which time the germ disc separates from the inner cell mass thereby forming the amniotic cavity. The cells lining this cavity proliferate to form the amniotic epithelium which gradually separates from the trophoblast. The mesenchyme of the amnion is derived from the extraembryonic mesoderm of the blastocyst and collagen deposition by the fibroblasts imparts tensile strength to the amnion.

The chorion is composed of trophoblast and mesoderm.81,82 The trophoblast of the blastocyst destroys the epithelial layer of the uterine wall enabling the blastocyst to implant. The trophoblastic mass increases and penetrates the small capillaries so it eventually communicates with the maternal circulation via villi. The chorion, with its villi and the maternal wall of the uterus form the placenta. The chorion and villi facing the uterine lumen are exposed to a less efficient blood supply and become reduced so that the chorion and the amnion eventually press together forming the amniochorion; an inner amniotic membrane of epithelial cells, mesenchymal cells and collagen, and an outer chorionic membrane of trophoblast, mesenchyme and maternal decidua. The mesenchymal layers of the amnion and chorion form an interface and are easily separated by dissection.

2 Favourable attributes of amniochorion and its use in reconstructive procedures

The amnion and the chorion, together or alone have been used for eighty years83 as wound dressings and grafts because of the tensile strength and low antigenicity.84,85 This foetal membrane was first used in 1910 by Davis86 for skin transplantation, then by Stern87 and Sabella88 in 1913 and Kubanyi in 1941-4889 as dressings for burned and ulcerated skin surfaces. They found that after 48 hours the amnion was adherent to the wound. The results
were good as there was no infection in clean wounds, there was a reduction in pain and the rate of re-epithelialization was greater than with traditional dressings. Douglas,\textsuperscript{90} studying the effectiveness of amnion and chorion as temporary wound coverings, found that chorionic grafts survived 13 days, encouraged neovascularization and that epithelial regeneration was complete by 14 days. Numerous investigators have reported that amniochorion as a surface graft was superior to allograft or xenograft skin because there was a decrease in infection, a decrease in fluid, protein, and electrolyte loss and a marked reduction in pain.\textsuperscript{91-94}

Infants have been treated with their own foetal membranes (autograft) in an attempt to repair abdominal wall defects.\textsuperscript{95} Amniochorion has also been used successfully to heal persistent wounds in diabetic patients\textsuperscript{96}, to treat chronic leg ulcers\textsuperscript{97}, to cover the peritoneal cavity following debridement for necrotizing cellulitis\textsuperscript{98}, and to treat vaginal aplasia\textsuperscript{99}. In the latter situation amnion was applied to the vaginal mould so that the mesenchyme protected the underlying granulation tissue.

Ammiochorion, as an allograft, is weakly antigenic\textsuperscript{84} and while the trophoblast forms an allogeneic interface with the maternal tissues, under normal circumstances the foetus is not rejected. This has been the object of much research during the last decade and investigators have found that trophoblast lacks or weakly expresses Major Histocompatibility Antigens HLA-A,B,C, responsible for rejection.\textsuperscript{100-104} The amnion and the chorion do appear to display some membrane antigens.

Human foetal membranes provided a temporary scaffold for regenerating urothelium in dogs and rabbits undergoing bladder reconstruction\textsuperscript{14,20} however, clinical trials have not been undertaken because the extent of bladder wall regeneration and fibrous contracture has not been adequately studied.
Isolated intestinal segments as well as grafts from the gastrointestinal tract retain their mucosa and their musculature so that mucus is continually produced and electrolytes in the urine are absorbed. The peristaltic contractions of the bowel cause incomplete bladder emptying and incontinence, reflux and infection are common. Other tissue grafts such as fascia, peritoneum and allograft bladder mucosa cause bone formation when used to cover bladder defects. Some of the synthetic grafts are absorbed before the bladder wall is reconstituted, while other synthetic grafts persist causing foreign body reactions and scar tissue formation. Prosthetic bladders are still being developed but one major consideration is the security of an anastomosis between the living tissue and a synthetic mould. Another consideration is the interaction of urine and the material used to construct the artificial bladder because stones readily form on foreign bodies in the bladder.

Three membranes presented in the literature appeared somewhat successful; omentum, amnionchorion and synthetic poly (alpha amino acid). Human amnionchorion is used in this present study because of its success as a biological dressing, its availability, and because of the encouraging results reported in previous animal studies. Human amnionchorion may be used clinically if results favoured this technique.

These membranes may be successful as grafts in bladder reconstruction because they meet the appropriate requirements. That is, they are resorbed slowly and therefore may allow bladder urothelium to regenerate before complete degradation of the grafts, as well, smooth muscle may regenerate, potentially imparting normal function and capacity.
Part Two: PURPOSE
I. PRELIMINARY STUDY: Amniochorion as a small patch-graft

The purpose of this study was to investigate the feasibility of using a small patch (2 cm²) of amniochorion to determine if it would survive as a graft in the bladder long enough to support the regeneration of the bladder wall. Cystography was used to detect possible leakage from the defect and histological examination was undertaken to document possible regeneration of urothelial and smooth muscle cells.

II. BLADDER RECONSTRUCTION: Single and multi-layered grafts

The purpose of this study was to investigate the effectiveness of amniochorion as a temporary tissue replacement or scaffold in bladder reconstruction. The following parameters were measured.

1. Bladder Capacity

The intent was to measure bladder capacities at post-operative intervals to determine whether the amniochorion grafts enabled the bladders to maintain a functional capacity and whether the healing process and/or immune response eventually produced a fibrotic, contracted bladder.

2. Immunologic Response

While amnionic membranes constitute a xenograft in the current study, the immunologic response had not been assessed following bladder reconstruction procedures in previous studies. The intent was to assess the ability of amniochorion to provide a temporary scaffold for bladder tissue which would regenerate to bridge the defect. It was necessary to study the foreign-body inflammatory reactions because of the effect on the healing process. It was also important to document whether these reactions produced tissue-urine interactions, the clinical importance being possible leakage and stone formation. This model also provided an opportunity to study the immunogenicity of an amniochorion xenograft.
3. Healing and Tissue Regeneration

Previous reports on the extent of smooth muscle regeneration following bladder reconstruction have been conflicting. The purpose of investigating this parameter was to document reconstitution of the bladder wall during a four month period and to assess the extent of urothelial and smooth muscle regeneration and collagen deposition.
Part Three: MATERIALS AND METHODS
I. Preparation of amniochorion grafts

Fresh, sterile human amniochorion was obtained at the time of uncomplicated Caesarean Section (Department of Obstetrics and Gynaecology at Grace Hospital, Vancouver, B.C.) and was washed with sterile saline to removed blood. It was stored in Hank’s solution at 4 °C until the time of surgery. Membranes stored longer than 4 h were rinsed in a 0.025% solution of sodium hypochlorite, rinsed four times in sterile saline.

II. Animal Preparation and Surgical Procedure

Fifty-six adult male New Zealand rabbits weighing 2.5 to 3.5 kg were divided into 6 groups: a preliminary study group (n=6), a control group (n=7), and three experimental groups in which bladders were reconstructed with a single layer of amniochorion (n=17), a double layer (n=14) and a quadruple layer (n=12).

The anaesthetic regime consisted of intramuscular pre-medication with Ketamine hydrochloride, 50 mg/kg (Rogarsetic, Rogar/STB, Montreal) and Acepromazine maleate, 0.25 mg/kg (Aceprozine, J.Webster, Downsview, Ontario), followed by a slow infusion of intravenous sodium pentobarbital, 100 mg (Somnotol, M.T.C. Pharmaceuticals, Mississauga, Ontario) and 0.20 mg atropine sulfate, (Abbott Laboratories, Montreal, Quebec) diluted with 1.5 mL of sterile saline, administered in 0.50 mL increments every 5 min until the desired depth of anaesthesia was attained. Animals were maintained on an intravenous drip of 0.9% NaCl to a maximum of 125 mL to compensate for the fluid and blood loss during surgery.

After sterile preparation of the abdomen the bladder was exposed through a midline abdominal incision. In the six rabbits comprising the preliminary study group a 2 cm² section of bladder dome was replaced with a graft of fresh sterile human amniochorion. The graft was sutured to the bladder wall with a single row of 5-0 polyglycolic acid suture then the abdomen was closed in 3 layers with 5-0 polyglycolic acid suture. The rabbits were followed for 1 month.
In the control groups and in the remaining three experimental groups, the bladder was exposed through an abdominal incision. Four corner sutures of 5-0 polyglycolic acid placed circumferentially around the the middle of the bladder marked the plane of excision and the anterio-superior aspect of the bladder was excised (Figure 1). Three control rabbits had the excised bladder dome resutured to the bladder with 7-0 chromic gut suture. The remaining 4 controls had the bladders closed without tissue replacement of the defect.

In the first experimental group, a single layer of amniochorion was placed with the amnion facing the bladder lumen, over an inflated Foley balloon within the bladder. The membrane was trimmed to match the shape of the defect then anchored with corner sutures (Figure 2). The graft, usually larger than the defect, was secured to the bladder wall with a water-tight running suture of 5-0 polyglycolic acid, which was later changed because of urinary extravasation through the needle and suture tracts to a double row of 7-0 chromic gut. In the second experimental group 2 layers of membranes were folded to form a double thickness with the amnion side out. The graft was attached to the bladder with 2 layers of suture; a full-thickness layer and a serosal layer both using 7-0 chromic gut. In the third experimental group, 4 layers of membrane comprised the graft, again 2 layers of sutures were used to ensure a leak-proof anastomosis. In the control and experimental groups the outer surface of the defect was outlined with black silk suture and metal clips (Figure 3). The abdomen was closed with 3 layers of 5-0 polyglycolic acid suture and an indwelling catheter kept the bladder drained for 2 days. The animals were followed for up to 16 weeks and were maintained on standard laboratory rabbit chow and water. Ampicillin (250 mg) was administered i.m. daily for 10 days.
Figure 1. Surgical Preparation. The bladder has been exposed and the amniochorion membranes have been washed with sterile saline.
Figure 2. Bladder Reconstruction. The top half of the bladder has been excised and a single layer of amniochorion has been sutured into place.
Figure 3. Autopsy Specimen of a Quadruple-layer Graft One Day After Surgery. In this anterio-superior view U marks the urethral end of the bladder and D marks the dome of the bladder. The increased thickness of the graft is immediately apparent when compared to Figure 2.
III. Parameters

1 Bladder capacity

Bladder capacities were measured pre-operatively, and at varying intervals post-operatively (from 2-17 weeks). Animals administered anaesthetic prior to urodynamic investigation have significantly higher bladder capacities compared to unanaesthetized animals. To study bladder capacities without the effect of anaesthesia, rabbits were subjected to immobility reflex which is a state of unresponsiveness resulting from repetitive stimulation, pressure on body parts, inversion and restraint. During the immobility reflex, animals tolerate minor procedures without motor response. In this study, the rabbit was grasped by his limbs and quickly placed in a supine position with limbs extended and held. By gently stroking the rabbits cheeks, immobility reflex was induced within 30 sec and maintained for the duration of the procedure.

Bladder capacities were measured by filling the bladder with physiologic saline (37 C) via a urethral catheter at a physiologic rate of 1.9 mL/min. Capacity was measured as the volume of urine at spontaneous voiding. The procedure was immediately repeated in all rabbits to verify the results.

2 Cystography

Radiographs were taken at 2 weeks and 4 weeks post-operatively and prior to sacrifice with a 20% solution of diatrizoate sodium (Hypaque sodium, Winthrop Laboratories, Aurora, Ontario). The X-rays were processed in a Picker automatic X-ray developer in the Department of Radiology at Vancouver General Hospital.
3 Immunology

a. Amniochorion graft degradation

Cryopreservation

Biopsies of the bladder/graft junction and the graft, obtained at sacrifice were immersed in methylbutane then placed in liquid nitrogen for 30 sec. The tissue was then stored in plastic tubes at -70 C.

Sectioning

The frozen tissue was warmed to -20 C and imbedded in O.C.T. Compound (Tissue-tek, Miles Scientific, Naperville, Illinoinis), on a metal chuck. The blocks were trimmed on a cryostat and 6 u sections were placed on slides, pretreated with a 1% solution of white household glue, (Elmers Glue-All, Borden, Columbus, Ohio). The sections were encircled with a diamond marking pencil.

Staining protocol for tissue immunofluorescence

The slides were washed in 3 changes of phosphate buffered saline (PBS) for a total of 10 min at room temperature, then fixed in cold high grade acetone at 4 C for 30 min. The rest of the procedures were carried out at room temperature. The tissues were rinsed and rehydrated in 3 changes of PBS for 10 min and excess fluid from around the circle was removed with a cotton gauze pad. Monoclonal mouse antihuman HLA-A,B,C, antibodies (Atlantic Antibody, Scarborough, USA) diluted 1:10 and 1:20 were reacted with the sections for 30 min. The slides were washed with 3 changes of PBS for 15 min. Goat antimouse IgG (H and L) conjugated to FITC (Zymed Laboratories, San Francisco) 1:10 and 1:20 were reacted with the sections for 30 min at room temperature. After a final wash of three changes of PBS for 15 min the slides were coverslipped and fluorescence was assessed with a Nikon fluorescent microscope (HFX-11). A piece of amniochorion, frozen at the time of surgery, served as the positive control and bladder tissue removed at the time of surgery served as the negative control. Tissues were also tested for autofluorescence.
b. Rabbit antihuman antibodies - Complement Dependent Cytotoxicity (CDC)

Blood was collected at the time of bladder reconstruction, two and four weeks postoperatively and at sacrifice. Clotted blood was then centrifuged at 400g for 10 min and the sera were aspirated into plastic tubes and stored at -70°C. All sera were screened, in triplicate, against a panel of normal human lymphocytes, in a complement-dependent cytotoxic (CDC) reaction, to ascertain the presence of antihuman antibodies. The screening was performed in the Immunology Laboratory at the Vancouver General Hospital under the direction of Dr. J. Denegri. The sera were reacted with human lymphocytes from 10 normal donors displaying the most common HLA-A,B,C, antigens. Further details of the protocol are as follows:

One microlitre of rabbit serum was transferred into each Terasaki typing microplates. Donor lymphocytes (1-2 x 10^3 cells in TC-199 medium) and 5% fetal calf serum were added. The plates were incubated at room temperature for 30 min then 5 microlitres of rabbit complement were reacted with the cells for 1 h at room temperature. Eosin (4 microlitres) was added for 2 min to stain the dead cells, then 8 microlitres of formalin (4%) was introduced to stop the reaction. The plates were read and scored according to the number of dead cells. (Table I)
Table I. Complement-dependent Cytotoxicity Test Using Human Lymphocytes - Scoring

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<tr>
<th>Percentage of dead cells</th>
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<td>80 - 100</td>
<td>8</td>
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<td>60 - 80</td>
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Dead cells stain with eosin and are scored accordingly in which a score of 6 to 8 represents a strong reaction while 2 to 4 represent a weak reaction.
c. Aliquots of sera from control and experimental rabbits, previously screened on the lymphocyte minipanel (CDC), underwent absorptions with platelets in an attempt to partially define antibody specificity. Platelet absorptions were used to remove antibodies to Class I antigens as well as other platelet-associated antigens, theoretically, leaving behind antibodies to amniochorion-associated antigens. These sera were then tested against the human lymphocyte panel to determine if antibodies cytotoxic to human lymphocytes were present. Sera were also reacted with human amniochorion in an indirect immunofluorescent technique to determine if antibodies to amniochorion were present (See section 'e').

Platelet Absorption: a platelet pellet from a collection of pooled platelets was built by repeated centrifugation at 3000g until a volume equal to the sera was established. The same volume of serum was added to the tube which was rotated gently end-over-end for 1 h. Final centrifugation at 3000g separated sera from the platelet pellet. The sera were divided into 2 equal aliquots for testing on the CDC lymphocyte minipanel and the immunofluorescent reaction with human amniochorion. The positive controls included sera which had not undergone absorption and the negative controls consisted of sera from control rabbits and sera from experimental rabbits prior to bladder reconstruction.

d. Aliquots of sera from control and experimental rabbits previously screened on the lymphocyte panel underwent absorption with amniochorion in an attempt to define antibody specificity. These absorptions attempted to remove antibodies associated with amniochorion, while leaving behind antibodies to HLA Class I antigens. These sera were also tested against the human lymphocyte panel in a CDC assay to ascertain the presence of antibodies cytotoxic to lymphocytes. Sera, as well, were reacted with human amniochorion to determine if antibodies generated against amniochorion were present (See section 'e').
Amniochorion absorption: pre-operative amniochorion was minced into small pieces (1 mm²) with iris scissors, then incubated in 40 ml of 0.3% collagenase made up in Ca²⁺, Mg²⁺ free PBS at room temperature for 30 min. The mixture was passed through a stainless steel screen (80 mesh/square inch) and centrifuged at 400g for 7 min. Approximately 2 x 10⁷ cells were harvested. An equal volume of rabbit serum was added to the cells and gently rotated end-over-end for 1 h. After centrifugation, the supernatant was subjected to CDC assays and to immunofluorescent studies on amniochorion (see below). The positive controls included sera which had not undergone absorption and negative controls consisted of sera from control rabbits and sera from experimental rabbits prior to bladder reconstruction.

e. Immunofluorescent reaction with human amniochorion: cryostat sections of pre-operative amniochorion were fixed in acetone at 4°C for 30 min, then washed and rehydrated in 3 changes of PBS, for 30 min at room temperature. Platelet-absorbed or amniochorion-absorbed sera were added to the slides and incubated for 30 min at room temperature. Slides were washed in 3 changes of PBS for 15 min then 1:10 dilution of goat antirabbit IgG conjugated to FITC was added for 30 min at room temperature. After a final wash with 3 changes of PBS, sections were assessed for fluorescence with a Nikon Fluorescent microscope. Positive controls consisted of sera excluded from the absorption procedure and negative controls of sera from control and experimental rabbits prior to surgery.

4 Histology and electron microscopy

Tissue biopsies were fixed in 10% buffered formalin phosphate for 4-8 hr and stored in 70% ethanol. The samples were first dehydrated through varying concentrations of alcohol and xylene then were embedded in paraffin. Sections were cut on a microtome, model 820 (American Optical, Buffalo, New York) and stained with H&E and Masson's trichrome stain (Lab-pac, Fisher Scientific, Vancouver, Canada). With Masson's, nuclei stain black, cytoplasm and muscle stain red and collagen stains blue.
Electron microscopy was performed in the Electron Microscopy suite at the Vancouver General Hospital under the direction of Dr. Noel Quenville. Glutaraldehyde-fixed specimens were fixed in 1% osmic acid, rinsed with distilled water, stained with uranyl acetate, rinsed, dehydrated then infiltrated with propylene and Effapoxy mixture. The tissues were then embedded in 100% Effapoxy mixture. Sections (60-100 nm) were stained with toluidine blue then placed on a grid stained with Reynolds lead citrate. Sections were viewed with a Zeiss EM CR 10 electron microscope (Zeiss, Oberkochen, West Germany).

5 Stone analysis

A small amount of crushed bladder calculi was analysed with an Oxford Stone Analysis Set for calcium, magnesium, carbonate, phosphate and ammonia. These analyses were carried out in the Clinical Chemistry Laboratory at the Vancouver General Hospital under the direction of Dr. W. Godolphin.
STATISTICAL ANALYSIS

1. Bladder Capacity

The paired t-test was used to determine significant differences between pre-operative and post-operative bladder capacities in control and experimental groups.

A two-way analysis of variance\textsuperscript{110} was used to determine significant differences between single, double and quadruple layer group means at varying intervals (<4 weeks, 5-8 weeks, >9 weeks). Bartlett's test for homogeneity of variance was applied to the data and when not significant (i.e., when variances were declared homogeneous) a two-way analysis of variance was performed. Analyses producing significant differences were subjected to the Duncan multiple comparison procedure to identify which specific groups were significantly different\textsuperscript{111}. The Duncan procedure compares differences between means with the 'least significant range' (Rp). Differences greater than this range are considered significant when p<0.05).
Part Four: RESULTS
I. PRELIMINARY STUDY: Amniochorion as a small patch-graft (2 cm²)

1. Radiology

   Continent bladders with no evidence of leakage, stone formation or graft extrusion were demonstrated radiographically at 4 weeks post-operative.

2. Gross Pathology

   The amniochorion graft could not be distinguished from the rest of the bladder upon gross inspection as connective tissue was present where the graft had once been. The luminal layer of the bladder appeared normal in 5 rabbits, but a small area of crystallization was detected on the luminal surface of the bladder in the sixth rabbit.

3. Histopathology

   Biopsies were obtained from the bladder, the bladder/graft junction and the grafted area. The bladder/graft junction was identified histologically by the presence of suture material and it was at this site that smooth muscle cells bridged the junction and infiltrated the grafted area. Despite complete urothelization of the graft site, granulation tissue and inflammatory cells, including macrophages, fibroblasts, and lymphocytes, comprised the greatest portion of the regenerated bladder wall. There was evidence of smooth muscle cell regeneration but organized bundles were not apparent 4 weeks after surgery. The amniochorion was not evident microscopically and may have undergone resorption.
II. BLADDER RECONSTRUCTION

Bladder capacities from 40 rabbits measured pre-operatively ranged from 16 to 121 mL with a mean of 50 ±4 ml (S.E.M.) (Figure 4). With a median of 42 mL the graph in figure 4 is considered positively skewed. As a result of the large standard deviation (25 mL), paired t-tests and analyses were performed only in rabbits with pre-operative and post-operative data.

1. Effect on Bladder Capacity

1.1 CONTROLS: Effect on Bladder Capacity

a. Pre-operative Capacity

Bladder capacities were measured in 5 control rabbits and were found to range from 34 - 51 ml as shown in Table II.

b. Capacity 4 weeks post-operatively

The capacity was reduced by 45-66% in two bladders assessed after 4 weeks in control group A (Figure 5). This represented a nonsignificant trend to decreased capacity (P=.06). The transected dome had been re-attached so loss of tissue was probably not responsible for the decreased capacity, however, reduced capacity in control group B by 55% was probably partially attributable to loss of tissue.

c. Capacity after 11 - 12 weeks

As noted in Table II capacities of control group A increased to 32-49 ml which represents a decrease from pre-operative values of only 4-9% compared to a 55-66% decrease after 4 weeks post-operative (P=.12). The capacities in control group B were still reduced by 20-38% (Figure 5), and these differences were significant at p<0.05 in a paired t-test.
FIGURE 4. Frequency histogram of bladder capacities before reconstruction.
Sample size = 40
Table II. Controls - Bladder Capacities Measured Before Reconstruction (Pre-op) and at Post-operative Intervals (Post-op)

<table>
<thead>
<tr>
<th>Control number</th>
<th>Pre-op (mL)</th>
<th>4 weeks (mL)</th>
<th>Post-op 11 - 12 weeks (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>35</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>2A</td>
<td>51</td>
<td>23</td>
<td>49</td>
</tr>
<tr>
<td>Mean</td>
<td>43</td>
<td>Mean 18</td>
<td>Mean 41</td>
</tr>
<tr>
<td>S.E.±</td>
<td>8</td>
<td>S.E.± 6</td>
<td>S.E.± 9</td>
</tr>
<tr>
<td>3B</td>
<td>44</td>
<td>-</td>
<td>35</td>
</tr>
<tr>
<td>4B</td>
<td>34</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>5B</td>
<td>47</td>
<td>21</td>
<td>29</td>
</tr>
<tr>
<td>Mean</td>
<td>42</td>
<td>Mean</td>
<td>29</td>
</tr>
<tr>
<td>S.E.±</td>
<td>4</td>
<td>S.E.±</td>
<td>3</td>
</tr>
</tbody>
</table>
FIGURE 5. Control groups A and B. Bladder capacity (mL) expressed as a percent change between pre-operative and post-operative capacities after 4 - 16 weeks.
0 - 4 weeks: -43 to -66%
9 -16 weeks: - 4 to -38%
1.2. SINGLE LAYER RECONSTRUCTION: Effect on Bladder Capacity

a. Pre-operative Capacity

Capacities determined before surgical reconstruction with a single layer of amniochorion ranged from 36 to 50 mL with a mean of 46 ±3mL (S.E.M.).

b. Post-operative Capacity at 2-4 weeks

Capacities assessed in 2 rabbits at 4 weeks were found to be reduced by 44% and 47% as can be seen in Figure 6. This difference was significant at p<0.05, using a t-test for paired values.

c. Post-operative Capacity after 5-8 weeks

Capacities, listed in Table IV, were found to range from 16 - 58 mL, with a mean of 35 ± 4 ml (S.E.M.). There were no significant differences (p<0.05) between pre-operative and post-operative capacities after 5 - 8 weeks in 6 bladders but one rabbit in this group did have a 68% reduction in capacity at this time. Two of the six bladders measured after 4 weeks had significantly smaller capacities, however, 4 weeks later the capacities had not only returned to normal but had increased (Figure 6).

d. Post-operative Capacity between 9 and 16 weeks

Bladder capacities ranged between 48 and 60 ml after 9 to 16 weeks as noted in Table V. There were no significant differences between the pre-operative mean, 47 ±3mL (S.E.M.) and the post-operative mean, 55 ±4mL (S.E.M.) at 9-16 weeks.
Table III. Single Layer - Bladder Capacities Measured Before Reconstruction (Pre-op) and at Post-operative Intervals of 4 Weeks or Less (Post-op).

<table>
<thead>
<tr>
<th>Single Layer</th>
<th>Pre-op (mL)</th>
<th>Post-op (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit #</td>
<td></td>
<td></td>
</tr>
<tr>
<td>281</td>
<td>36</td>
<td>20</td>
</tr>
<tr>
<td>286</td>
<td>38</td>
<td>20</td>
</tr>
<tr>
<td>Mean</td>
<td>37</td>
<td>Mean 20</td>
</tr>
<tr>
<td>S.E.±</td>
<td>1</td>
<td>S.E.± 0</td>
</tr>
</tbody>
</table>
Table IV. Single Layer - Bladder Capacities Measured Before Reconstruction (Pre-op) and at Post-operative Intervals of 5-8 Weeks (Post-op).

<table>
<thead>
<tr>
<th>Single Layer</th>
<th>Pre-op mL</th>
<th>Post-op mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit #</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7667</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>8653</td>
<td>50</td>
<td>38</td>
</tr>
<tr>
<td>609</td>
<td>46</td>
<td>40</td>
</tr>
<tr>
<td>7512</td>
<td>40</td>
<td>36</td>
</tr>
<tr>
<td>286</td>
<td>38</td>
<td>42</td>
</tr>
<tr>
<td>281</td>
<td>36</td>
<td>40</td>
</tr>
<tr>
<td>Mean</td>
<td>43</td>
<td>Mean 35</td>
</tr>
<tr>
<td>S.E.±</td>
<td>3</td>
<td>S.E.± 4</td>
</tr>
</tbody>
</table>
Table V. Single Layer - Bladder Capacities Measured Before Reconstruction (Pre-op) and at Post-operative Intervals of 9 - 16 Weeks (Post-op).

<table>
<thead>
<tr>
<th>Single Layer</th>
<th>Pre-op (mL)</th>
<th>Post-op (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit #</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8653</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>7667</td>
<td>50</td>
<td>48</td>
</tr>
<tr>
<td>7512</td>
<td>40</td>
<td>56</td>
</tr>
<tr>
<td>Mean</td>
<td>47</td>
<td>Mean 55</td>
</tr>
<tr>
<td>S.E.±</td>
<td>3</td>
<td>S.E.± 4</td>
</tr>
</tbody>
</table>
FIGURE 6. Single-layer Reconstruction. Bladder capacity (mL) expressed as a percent change. A comparison between pre-operative capacities and post-operative capacities after 4 - 16 weeks.
0 - 4 weeks: -47 to -44%
5 - 8 weeks: -68 to +11%
9 - 16 weeks: -10 to +40%
1.3. DOUBLE LAYER RECONSTRUCTION: Effect on Bladder Capacity

a. Pre-operative Capacities

The normal bladder capacity, prior to reconstruction, ranged from 16 to 104 mL with a mean of 46±13 mL (S.E.M.).

b. Post-operative Capacities after 2 to 4 weeks

The bladder capacities, shown in Table VI, ranged from 6 to 19 mL, a reduction of 20 to 81% (Figure 7). According to the paired t-test this reduction tended toward statistical significance as (P=.06) compared to pre-operative capacities.

c. Post-operative Capacities after 5 to 8 weeks

Post-operative capacities were not significantly different from those measured pre-operatively as depicted in Table VII. The values ranged from 16 to 80 mL representing a percent change of -33 to -16 (Figure 7).

d. Post-operative Capacities After 9 - 16 weeks

The bladder capacities ranged from 26 to 47 mL (Table VIII). The capacity of one bladder was decreased by 62%, while the capacities of two others were increased by 113 and 119% but as a group, these differences were not significant using the paired t-test (p<0.05).
Table VI. Double Layer - Bladder Capacities Measured Before Reconstruction (Pre-op) and at Post-operative Intervals of 2 to 4 Weeks (Post-op).

<table>
<thead>
<tr>
<th>Double Layer</th>
<th>Pre-op (mL)</th>
<th>Post-op (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit#</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1034</td>
<td>70</td>
<td>13</td>
</tr>
<tr>
<td>822</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>801*</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td>801*</td>
<td>24</td>
<td>19</td>
</tr>
<tr>
<td>2020</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>881</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>Mean</td>
<td>29</td>
<td>Mean 10</td>
</tr>
<tr>
<td>S.E.±</td>
<td>8</td>
<td>S.E.± 2</td>
</tr>
</tbody>
</table>

* Rabbit #801: Capacity measured after 2 weeks was 8 mL and after 4 weeks was 19 mL.
Table VII. Double Layer - Bladder Capacities Measured Before Reconstruction (Pre-op) and at Post-operative Intervals of 5 to 8 Weeks (Post-op).

<table>
<thead>
<tr>
<th>Double Layer</th>
<th>Pre-op (mL)</th>
<th>Post-op (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit #</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1210</td>
<td>104</td>
<td>80</td>
</tr>
<tr>
<td>812</td>
<td>87</td>
<td>73</td>
</tr>
<tr>
<td>822</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>Mean</td>
<td>72</td>
<td>Mean</td>
</tr>
<tr>
<td>S.E.±</td>
<td>24</td>
<td>S.E.±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
</tr>
</tbody>
</table>
Table VIII. Double Layer - Bladder Capacities Measured Before Reconstruction (Pre-op) and at Post-operative Intervals of 9 - 16 Weeks (Post-op).

<table>
<thead>
<tr>
<th>Rabbit#</th>
<th>Pre-op (mL)</th>
<th>Post-op (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1034</td>
<td>70</td>
<td>26</td>
</tr>
<tr>
<td>1043</td>
<td>22</td>
<td>47</td>
</tr>
<tr>
<td>881</td>
<td>16</td>
<td>35</td>
</tr>
<tr>
<td>Mean</td>
<td>36</td>
<td>Mean 36</td>
</tr>
<tr>
<td>S.E.±</td>
<td>17</td>
<td>S.E.± 6</td>
</tr>
</tbody>
</table>
FIGURE 7. Double-layer Reconstruction. Bladder capacity (mL) expressed as a percent change. A comparison between pre-operative capacities and post-operative capacities after 4 - 16 weeks.

0 - 4 weeks: -20 to -81%
5 - 8 weeks: -33 to -16%
9 - 16 weeks: -62 to +119%
1.4. QUADRUPLE LAYER CLOSURE: Effect on Bladder Capacity

a. Pre-operative Bladder Capacities

Bladder capacities ranged from 25 to 102 mL with a mean of 56 ± 9mL(S.E.M.).

b. Post-operative capacities at 2-4 weeks

Capacities measured after 2 to 4 weeks were significantly reduced according to the paired t-test (p<0.05) (Table IX). Figure 8 graphically demonstrates this reduction in which capacities were 32 - 86% lower than those measured pre-operatively.

c. Post-operative capacities at 5-8 weeks

The bladder capacities ranged from 21 to 34 mL but these values were not significantly reduced from pre-operative values (p< 0.05) as seen in Table X. The percent changes (Figure 8) between pre-operative and post-operative capacities were not as great as those seen after 2-4 weeks.

d. Post-operative Capacities at 9 - 16 weeks

Bladder capacities at 16 weeks post-operatively were not significantly different from pre-operative values (Table XI). An initial reduction in bladder size during the early post-operative period appeared to be a temporary phenomenon as capacities returned to normal after several weeks. This effect was also seen in the Single and Double-Layer experimental groups.
Table IX. Quadruple Layer - Bladder Capacities Measured Before Reconstruction (Pre-op) and at Post-operative Intervals of 2 to 4 Weeks (Post-op).

<table>
<thead>
<tr>
<th>Quadruple Layer</th>
<th>Pre-op (mL)</th>
<th>Post-op (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit #</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1686</td>
<td>102</td>
<td>54</td>
</tr>
<tr>
<td>1135</td>
<td>82</td>
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<tr>
<td>1097</td>
<td>73</td>
<td>10</td>
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<td>1378</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>Mean</td>
<td>71</td>
<td>Mean 25</td>
</tr>
<tr>
<td>S.E.±</td>
<td>16</td>
<td>S.E.± 10</td>
</tr>
</tbody>
</table>
Table X. Quadruple Layer - Bladder Capacities Measured Before Reconstruction (Pre-op) and at Post-operative Intervals of 5 - 8 Weeks (Post-op).

<table>
<thead>
<tr>
<th>Quadruple Layer</th>
<th>Pre-op (mL)</th>
<th>Post-op (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit #</td>
<td></td>
<td></td>
</tr>
<tr>
<td>390</td>
<td>80</td>
<td>34</td>
</tr>
<tr>
<td>1160</td>
<td>59</td>
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<td>1095</td>
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<td>1360</td>
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<td>25</td>
</tr>
<tr>
<td>Mean</td>
<td>48</td>
<td>Mean 27</td>
</tr>
<tr>
<td>S.E.±</td>
<td>9</td>
<td>S.E.± 3</td>
</tr>
</tbody>
</table>
Table XI. Quadruple Layer - Bladder Capacities Measured Before Reconstruction (Pre-op) and at Post-operative Intervals of 9 - 16 Weeks (Post-op).

<table>
<thead>
<tr>
<th>Quadruple Layer</th>
<th>Pre-op (mL)</th>
<th>Post-op (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit # 1135</td>
<td>82</td>
<td>80</td>
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<td>390</td>
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<td>1097</td>
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<td>11</td>
<td>S.E.±</td>
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<td></td>
<td>48</td>
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<tr>
<td></td>
<td></td>
<td>11</td>
</tr>
</tbody>
</table>
FIGURE 8. Quadruple-layer Reconstruction. Bladder capacity (mL) expressed as a percent change. A comparison between pre-operative capacities and post-operative capacities after 4-16 weeks.
0 - 4 weeks: -79 to -32%
5 - 8 weeks: -63 to -11%
9 -16 weeks: -45 to -2.5%
ANALYSIS OF VARIANCE

A two-way analysis of variance was used to compare the pre-operative capacities and the post-operative capacities of the different experimental groups at the various post-operative intervals.

The variances between the groups were homogeneous according to the Bartlett test. A two-way analysis of variance revealed significant differences as a result of the various layers used and as a result of the post-operative interval. According to the Duncan multiple range tests, when only the layers were considered (regardless of the post-operative interval) capacities of the quadruple-layer groups were significantly smaller than those of the single- or double-layer groups. When only the post-operative intervals were assessed capacities at 0-4 weeks were significantly less than those at 9-16 weeks. The only significant difference found, when both layers and post-operative intervals were analysed together according to the Duncan multiple range test, was between capacities of the quadruple-layer group at a post-operative interval of 4 weeks or less and capacities of the single-layer group at 5-8 and 9-16 weeks and of the double-layer groups at 9-16 weeks. The interpretation drawn from these analyses is that the number of layers comprising the graft influenced the change in bladder capacities when differences in post-operative intervals were ignored. There were no significant differences between the various layers when similar post-operative intervals were compared. As noted previously with the paired t-test, significant differences were found in all experimental groups at post-operative intervals of 4 weeks or less. Differences in capacities were not significant for 5-8 or 9-16 week post-operative intervals.
II. BLADDER RECONSTRUCTION

2. Immunologic Response

2.1. CONTROLS: Immunologic Response

a. Immunofluorescent Detection of Amniochorion Graft

Cryostat sections of bladder tissue from control rabbits did not fluoresce when reacted with mouse antihuman HLA-A,B,C, antibodies and goat antimouse IgG conjugated to FITC.

b. Rabbit Antihuman Antibodies - Complement Dependent Cytotoxicity (CDC) Assay

Sera from control rabbits, screened on a minipanel of human donor lymphocytes in a complement-dependent cytotoxicity (CDC) test, failed to react indicating an absence of rabbit antibodies cytotoxic to human lymphocytes.

2.2. SINGLE LAYER: Immunologic Response

a. Immunofluorescent Detection of Amniochorion Graft

Tissue within the grafted portion of the bladder weakly fluoresced suggesting the presence of amniochorion. This weak positive fluorescence resulted when the tissues were reacted with mouse antihuman HLA-A,B,C, antibodies and goat antimouse IgG conjugated to FITC. Sections from the centre of the defect, in 2 bladders tested at 9 weeks, weakly fluoresced whereas sections from the bladder/graft junction did not fluoresce at all.

b. Rabbit Antihuman Antibodies - CDC Assay

All experimental rabbits (except one tested at 12 weeks) produced anti-human antibodies as evidenced in a complement-dependent cytotoxic reaction using the donor lymphocyte mini-panel (Table XII). Weak and negative reactions reported 1 week after surgery may reflect a lag in antibody production following antigenic challenge. Sera collected between 2 and 9 weeks generally scored high. Fifty-seven percent of the sera reacted with 9/10 or 10/10 of the wells of lymphocytes.
from the panel and of these high scores (usually 6 and 8), 88% occurred at intervals of 8 weeks or less. Decreased reactivity noted after 12-16 weeks may represent decreasing specificity and/or intensity. Allotypic differences between the lymphocyte-associated antigens of the CDC assay and the amniochorion-associated antigens of the graft would prevent antihuman antibodies from recognizing the antigens resulting in a negative CDC reaction. A lack of response probably did not result from a species failure to mount an immune response because the other rabbits in this group did generate antibodies cytotoxic to lymphocyte-associated antigens.
Table XII. Single-layer Reconstruction: Detection of Cytotoxic Anti-human Antibodies in Rabbit Sera Using a Complement-dependent Cytotoxicity (CDC) Reaction.

<table>
<thead>
<tr>
<th>Specimen Identification</th>
<th>Weeks Post-op</th>
<th>Strong Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>286</td>
<td>1</td>
<td>0/10</td>
</tr>
<tr>
<td>281</td>
<td>1</td>
<td>1/10</td>
</tr>
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<td>286</td>
<td>2</td>
<td>10/10</td>
</tr>
<tr>
<td>281</td>
<td>2</td>
<td>10/10</td>
</tr>
<tr>
<td>958</td>
<td>3</td>
<td>10/10</td>
</tr>
<tr>
<td>330</td>
<td>7</td>
<td>9/9</td>
</tr>
<tr>
<td>286</td>
<td>7</td>
<td>9/9</td>
</tr>
<tr>
<td>281</td>
<td>8</td>
<td>10/10</td>
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<tr>
<td>958</td>
<td>8</td>
<td>10/10</td>
</tr>
<tr>
<td>330</td>
<td>9</td>
<td>9/9</td>
</tr>
<tr>
<td>8653</td>
<td>12</td>
<td>0/10</td>
</tr>
<tr>
<td>7512</td>
<td>12</td>
<td>6/10</td>
</tr>
<tr>
<td>7667</td>
<td>12</td>
<td>6/10</td>
</tr>
<tr>
<td>7512</td>
<td>16</td>
<td>5/10</td>
</tr>
</tbody>
</table>

In a Complement-dependent Cytotoxicity (CDC) Reaction using a human donor lymphocyte panel (10 donors representing the major HLA-A,B,C, antigens), the numerator represents the number of strong positive reactions and the denominator represents the total number of possible reactions (donors).

A CDC reaction is considered a strong positive when >60% of the human donor lymphocytes are killed (scores 6-8) and subsequently stain with eosin.
c. Platelet-Absorbed Rabbit Sera

i) Complement-dependent cytotoxicity assay: platelets normally display antigens including HLA-A,B,C, transplantation antigens. Platelet-absorbed sera from 1 of 3 rabbits, produced a strong positive cytotoxic response to human lymphocytes (Table XV). This indicated that antibodies had been generated against antigens associated with lymphocytes and that these antibodies had not been removed by platelet absorption which may be explained by allotypic differences between the platelets used in the absorption protocol and the amniochorion graft. The antibodies, generated by the rabbits to the graft, perhaps did not recognize the corresponding but allogenically different antigens known also to exist on the lymphocytes (HLA Class I and TLX). The other two rabbits failed to produce strong responses but had reacted strongly in this test before platelet absorption so antibodies generated against Class I antigens and platelet-lymphocyte-associated antigens may have been effectively removed (Table XV).

ii) Immunofluorescent cryostat sections of amniochorion: platelet-absorbed sera were reacted with cryostat sections of human amniochorion frozen at the time of surgery. Goat antirabbit IgG conjugated to FITC produced a fluorescent response in all sections tested (Figure 9 and Table XVI).

d. Amniochorion-Absorbed Rabbit Sera

i) Complement-dependent cytotoxicity assay: serum from the one rabbit tested, produced a positive response, suggesting the presence of antihuman cytotoxic antibodies to antigens expressed on lymphocytes (Table XV).

ii) Immunofluorescent cryostat sections of amniochorion: no immunofluorescence could be detected in the sections tested. These results were contrary to those described above following platelet absorption, and may suggest that antibodies were produced with specificity to amniochorion (Figure 10 and Table XVI).
2.3. DOUBLE LAYER RECONSTRUCTION: Immunologic Response

a. Immunofluorescent Detection of Amniochorion Graft

All rabbit bladders assessed for the presence of human amniochorion reacted weakly to mouse antihuman HLA-A,B,C,- goat antimouse conjugated to FITC. Cryostat sections obtained at less than 9 weeks demonstrated slightly more intense fluorescence so they may have contained fragments of human tissue. However, conclusions from this technique could not be made because of the weak response which may have been the result of low antibody specificity or weak HLA expression of the amniochorion.

b. Rabbit Anti-human Antibodies - Complement-dependent Cytotoxicity

All rabbits produced antibodies to human tissue as documented by a complement-dependent cytotoxicity test using the human donor lymphocyte panel as seen in Table XIII. Seventy-two percent of the reactions were strong and 88% of these occurred after 2-8 weeks. The decreased response with time may have reflected a decreased antibody specificity and/or intensity.
Table XIII. Double-layer Reconstruction: Detection of Cytotoxic Anti-human Antibodies in Rabbit Sera Using a Complement-dependent Cytotoxic (CDC) reaction.

<table>
<thead>
<tr>
<th>Specimen Identification</th>
<th>Weeks</th>
<th>Strong Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Post-op</td>
<td></td>
</tr>
<tr>
<td>1165</td>
<td>2</td>
<td>5/10</td>
</tr>
<tr>
<td>1043</td>
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<td>6/10</td>
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<td>1210</td>
<td>2</td>
<td>10/10</td>
</tr>
<tr>
<td>2020</td>
<td>2</td>
<td>10/10</td>
</tr>
<tr>
<td>801</td>
<td>3</td>
<td>10/10</td>
</tr>
<tr>
<td>822</td>
<td>3</td>
<td>10/10</td>
</tr>
<tr>
<td>881</td>
<td>3</td>
<td>10/10</td>
</tr>
<tr>
<td>1043</td>
<td>4</td>
<td>6/10</td>
</tr>
<tr>
<td>812</td>
<td>4</td>
<td>9/10</td>
</tr>
<tr>
<td>1210</td>
<td>4</td>
<td>10/10</td>
</tr>
<tr>
<td>1165</td>
<td>4</td>
<td>10/10</td>
</tr>
<tr>
<td>1034</td>
<td>5</td>
<td>9/10</td>
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<tr>
<td>7111</td>
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<td>10/10</td>
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<td>5</td>
<td>10/10</td>
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<tr>
<td>822</td>
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<td>10/10</td>
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<td>881</td>
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<td>10/10</td>
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<tr>
<td>801</td>
<td>7</td>
<td>10/10</td>
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<td>1043</td>
<td>8</td>
<td>0/10</td>
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<td>9/10</td>
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<td>10/10</td>
</tr>
<tr>
<td>6875</td>
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<td>10/10</td>
</tr>
<tr>
<td>6875</td>
<td>16</td>
<td>9/10</td>
</tr>
<tr>
<td>881</td>
<td>17</td>
<td>0/10</td>
</tr>
</tbody>
</table>

In a Complement-dependent Cytotoxicity (CDC) reaction using a human donor lymphocyte panel (10 donors representing major HLA-A,B,C, antigens), the numerator represents the number of strong positive reactions and the denominator represents the total number of possible reactions (donors).

A CDC is considered strongly positive when >60% of the human lymphocytes are killed (scores 6 - 8) and subsequently stain with eosin.
c. Platelet-absorbed Rabbit Sera

i) Complement-dependent cytotoxicity: sera from 3 of 4 rabbits, produced positive responses when tested for complement-dependent cytotoxicity (Table XV). The positive reactions suggested the additional production of antihuman antibodies to antigens other than HLA-A,B,C, which may have been associated with human lymphocytes. Positive results may have also occurred because the cytotoxic antibodies may not have been removed during platelet absorption due to allotypic differences between amniochorion-associated antigens and platelet-associated antigens as reported with the single-layer group. One rabbit at 2 weeks did not respond but had responded positively before platelet absorption so the antibodies may have been effectively removed.

ii) Immunofluorescent cryostat sections of amniochorion: platelet-absorbed sera reacted with a piece of amniochorion, frozen at the time of surgery, and goat-antirabbit IgG conjugated to FITC to produce brilliant fluorescence in all sections (Table XVI).

d. Amniochorion-absorbed Rabbit Sera

i) Complement-dependent cytotoxicity: sera from the three rabbits tested produced positive responses, suggesting the presence of antihuman cytotoxic antibodies to antigens expressed on lymphocytes.

ii) Immunofluorescent cryostat sections of amniochorion: Negative responses were noted in the sections tested and may suggest that some of the antibodies generated against human tissue were specific to amniochorion and were removed during amniochorion absorption.
2.4. QUADRUPLE LAYER: Immunologic Response

a. Immunofluorescent Detection of Amniochorion Graft

Examination of cryostat sections of bladder, treated with mouse anti-human HLA-A,B,C, antibodies and goat anti-mouse IgG conjugated to FITC, revealed some focal areas of weak fluorescence. As reported for single and double-layer groups remnants of amniochorion may have accounted for these results.

b. Rabbit Anti-human Antibodies - Complement-dependent cytotoxicity

All rabbits in this group reacted positively in the complement-dependent cytotoxicity test as depicted in Table XIV. Nine of ten rabbits displayed a strong positive response after 2 weeks while sera from 1 rabbit did not attain a maximal score until 7. Seventy-seven percent of the measurements were 10/10 and 83% of these high scores occurred after 2-8 weeks after which time decreased reactivity was noted.
Table XIV. Quadruple-layer Reconstruction: Detection of Anti-human Antibodies in Rabbit Sera Using a Complement-dependent Cytotoxic (CDC) Reaction.

<table>
<thead>
<tr>
<th>Specimen Identification</th>
<th>Weeks Post-op</th>
<th>Strong Reaction</th>
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</thead>
<tbody>
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<td>1097</td>
<td>2</td>
<td>3/10</td>
</tr>
<tr>
<td>90</td>
<td>2</td>
<td>10/10</td>
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<tr>
<td>875</td>
<td>2</td>
<td>10/10</td>
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<tr>
<td>1360</td>
<td>2</td>
<td>10/10</td>
</tr>
<tr>
<td>1135</td>
<td>2</td>
<td>10/10</td>
</tr>
<tr>
<td>1208</td>
<td>2</td>
<td>10/10</td>
</tr>
<tr>
<td>1381</td>
<td>2</td>
<td>10/10</td>
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<td>1160</td>
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<td>10/10</td>
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<tr>
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<td>16</td>
<td>10/10</td>
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<tr>
<td>390</td>
<td>16</td>
<td>10/10</td>
</tr>
</tbody>
</table>

In a complement-dependent cytotoxicity (CDC) reaction using a human donor lymphocyte panel (10 donors representing major HLA-A,B,C, antigens), the numerator represents the number of strong positive reactions and the denominator represents the total number of possible reactions (donors tested).

A CDC is considered a strong positive when >60% of the lymphocytes are killed (scores 6 and 8) and subsequently stain with eosin.
c. Platelet-absorbed Rabbit Sera

i) Complement-dependent cytotoxicity: rabbit sera absorbed with platelets produced variable cytotoxic responses to human lymphocytes. Six of eight sera displayed strong responses and as noted previously may indicate the presence of antihuman antibodies which were not removed during the absorption procedure. Allotypy between the amniochorion-associated antigens and the platelet-associated antigens would result in ineffective absorption as discussed previously. The presence of a trophoblast/lymphocyte crossreactive antibody may also have caused the positive CDC response. The responses of the remaining two samples, which did not react or reacted weakly, suggested that antibodies were removed and that allotypy may not have existed in these two cases (Table XV).

ii) Immunofluorescent cryostat sections of amniochorion: platelet-absorbed rabbit sera reacted positively when tested with amniochorion and goat anti-rabbit IgG conjugated to FITC. All 9 sections showed intense fluorescence. The fluorescing structures appeared to be cells or tissue remnants, as noted previously in the single and double layer groups (Table XVI).

d. Amniochorion-absorbed Rabbit Sera

i) Complement-dependent cytotoxicity: sera from all rabbits tested, produced strong positive responses, as noted with the other experimental groups as well, indicating antihuman antibodies cytotoxic to lymphocyte-associated antigens.

ii) Immunofluorescent cryostat sections of amniochorion: a negative response was noted and was similar to that described for single and double-layer groups. Again these results may suggest that antihuman antibodies which were removed during the absorption protocol were specific for amniochorion.
Table XV. A Comparison of CDC Reactions Between Platelet-absorbed Sera and Amniochorion-absorbed Sera.

<table>
<thead>
<tr>
<th>Specimen Number</th>
<th>Platelet-absorbed Antisera</th>
<th>Amniochorion-absorbed Antisera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single Layer</td>
<td></td>
</tr>
<tr>
<td>7667</td>
<td>1/10</td>
<td>10/10</td>
</tr>
<tr>
<td>286</td>
<td>0/10</td>
<td>*</td>
</tr>
<tr>
<td>281</td>
<td>7/10</td>
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<tr>
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<td>Double Layer</td>
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<tr>
<td>1043</td>
<td>0/10</td>
<td>10/10</td>
</tr>
<tr>
<td>881</td>
<td>9/10</td>
<td>10/10</td>
</tr>
<tr>
<td>801</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>1034</td>
<td>10/10</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Quadruple Layer</td>
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<td>1160</td>
<td>0/10</td>
<td>10/10</td>
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<tr>
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<td>10/10</td>
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<tr>
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<td>1097</td>
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<td>10/10</td>
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<td>10/10</td>
</tr>
<tr>
<td>1360</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>875</td>
<td>10/10</td>
<td>10/10</td>
</tr>
</tbody>
</table>

CDC - Complement-dependent Cytotoxicity

* Insufficient sera for testing.

In a complement-dependent cytotoxicity reaction using a human donor lymphocyte panel, the numerator represents the number of strong positive reactions and the denominator represents the total number of possible reactions (10).

A CDC response is considered a strong positive when >60% of cells are killed (scores 6 and 8) and subsequently stain with eosin.
Table XVI. A Comparison of Tissue Immunofluorescence Between Platelet-absorbed and Amniochorion-absorbed Sera.

<table>
<thead>
<tr>
<th>Specimen Number</th>
<th>Amniochorion-absorbed Sera</th>
<th>Platelet-absorbed Sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single layer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7661</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>7667</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Double layer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>801</td>
<td>0</td>
<td>+++</td>
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<td>1043</td>
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<td>881</td>
<td>+</td>
<td>+++</td>
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<tr>
<td>Quadruple layer</td>
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<td></td>
</tr>
<tr>
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<td>0</td>
<td>+++</td>
</tr>
<tr>
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<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>875</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

* Cryopreserved amniochorion

0       no fluorescent response
+       nil to weak fluorescent response (may be background fluorescence)
+++     strong fluorescent response
FIGURE 9. Cryopreserved section of human amniochorion (160x) (rabbit #7667). This tissue was reacted with platelet-absorbed rabbit antiserum and goat-antirabbit IgG conjugated to FITC. Fluorescence is evident throughout the section.
FIGURE 10. Cryopreserved section of human amniochorion (160x) (rabbit #7667). This tissue was reacted with amniochorion-absorbed rabbit antiserum and goat-anti-rabbit IgG conjugated to FITC. The fluorescence noted in Fig. 11 is lacking in this section.
Summary of Immunologic Response to Xenograft

Portions of reconstructed bladders weakly fluoresced which may have indicated low antibody affinity or weak expression of HLA Class I antigens. Remnants of amniochorion may have persisted for more than 9 weeks but it was difficult to draw any conclusions from this test.

The presence of an immune response was documented by the complement-dependent cytotoxicity assay utilizing human donor lymphocytes. Rabbit sera contained antihuman antibodies which were cytotoxic to human lymphocytes and as illustrated in Figure 11, wide spread reactivity was evident in all three experimental groups. Eighty-eight percent of the strong reactivities occurred with sera obtained at post-operative intervals of 8 weeks or less and only 12% were from sera collected after 9 weeks. These results may indicate a decreasing lymphocytotoxic antibody titre. Eighty-two percent of the sera tested from the double-layer group and 77% of sera from the quadruple-layer group, showed strong positive reactivity (score of 6-8) with 9/10 or 10/10 of the panel members. Within the single-layer group only 57% of the sera reacted similarly, a difference which may be attributed to the smaller antigen dose comprising the single-layer graft.

Specificity of these antihuman antibodies however was unknown so moderately sensitive tests were undertaken to attempt to answer this: platelet-absorbed sera tested against a CDC lymphocyte panel and reacted with amniochorion tissue; amniochorion-absorbed sera reacted on a CDC lymphocyte panel and reacted with amniochorion.

Platelet-absorption

CDC Assay - Some of the sera which underwent platelet-absorption, to remove HLA, A,B,C, antibodies, produced a negative response on the CDC panel and some produced a positive response (Table XVII). The negative responses could have resulted from effective removal of cytotoxic antibodies by platelet absorption. Approximately 25 - 66% of the sera from the experimental groups
failed to react but had reacted positively before platelet-absorption. This indicated that some of the antibodies directed towards lymphocyte-associated antigens (perhaps HLA antigens) had indeed been removed. The positive responses could have resulted from platelet-absorption which was ineffective in removing all HLA antibodies and others associated with platelets because: the platelets were from a non-selected group so all of the HLA A,B,C, antigens may not have been represented; or allotypic differences between the platelets and the amniochorion graft would prevent the antibodies from reacting with the platelet-associated antigens. An additional check for platelet-absorption specificity and efficiency (HLA antibody removal) would have been to run an immunodiffusion gel using the platelet-absorbed antisera and HLA antigen and/or titrate the antibody before and after absorption. Another reason for the CDC positive results is that antibodies may have been generated against a trophoblast antigen which is also found on lymphocytes. In the literature such an antigen has been identified and has been called the trophoblast/lymphocyte crossreactive antigen.

Immunofluorescence - A positive response of platelet-absorbed antisera with amniochorion tissue, suggested that antibodies which reacted with amniochorion-associated antigens were present and had not been removed during platelet-absorption (Table XVII).

Amniochorion Absorption

CDC Assay - Rabbit sera, absorbed with amniochorion reacted positively in a CDC test as summarized in Table XVII, suggesting the presence of cytotoxic antibodies to human lymphocytes.

Immunofluorescence - Rabbit sera, absorbed with amniochorion, then reacted with amniochorion tissue failed to produce significant fluorescence, suggesting that antibodies removed in the amniochorion absorptions were specific to amniochorion (Table XVII) and that MHC antigens, if expressed are in low density.
FIGURE 11. Percentage distribution of responses for single, double and quadruple-layer reconstructions. The CDC (Complement-dependent Cytotoxicity) response is based on the number of strong cytotoxic reactions obtained when antihuman rabbit sera is incubated with human lymphocytes and complement. There are 10 lymphocyte donors per panel so the absissa represents the number of strong responses out of a maximum of 10.
Table XVII. Immunologic Response Summarized. Platelet-absorbed or Amniochorion-absorbed Rabbit Antisera Tested in Either a Complement-dependent Cytotoxicity (CDC) Reaction or an Indirect Immunofluorescent (I-F) reaction.

<table>
<thead>
<tr>
<th>Rabbit Sera</th>
<th>CDC</th>
<th>I-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-op sera</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(negative control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-op sera: no absorptions</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(positive control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-op sera: Platelet-absorption</td>
<td>- &amp; +</td>
<td>+</td>
</tr>
<tr>
<td>Post-op sera: Amniochorion-absorption</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+ positive response
- negative response
II. BLADDER RECONSTRUCTION

3. Healing and Tissue Regeneration

3.1. CONTROLS- Healing and Tissue Regeneration

The reconstructed bladders of the control groups appeared normal upon gross inspection with no evidence of anatomical distortion. Microscopically, granulation tissue filled the defect, fibroblasts and myofibroblasts were abundant and acute inflammatory cells were present at the wound margins and surrounding remnants of suture material (Figure 14). By 4 weeks urothelium had regenerated and finger-like extensions of smooth muscle, which appeared to originate from the wound margin, infiltrated the granulation tissue (Figure 15).

Biopsies obtained 11 weeks post-operatively showed complete healing and normal cellular architecture and histological differences between the two control groups were not apparent.
FIGURE 12. Section of control bladder (3 weeks post operative) stained with Masson’s (96x). The transected portion was reattached. Suture material (Su) was seen at the bladder/graft junction. Note the normal urothelium (U) and smooth muscle bundles (Sm) at the margin and granulation tissue (Gt) at the site of the defect.
FIGURE 13. Section of control bladder transected then closed (4 weeks post-operative, stained with Masson's) (25x). Remnants of suture (Su) are evident in the lower right corner. Abundant granulation tissue (Gt) is seen in the defect area. Smooth muscle (Sm) can be seen infiltrating the junction and urothelium (U) is undergoing regeneration.
3.2. EXPERIMENTAL GROUPS:

a. Gross Pathology

Examination of bladders from all experimental groups at 3-4 weeks post-operatively revealed white fibrous tissue extending from the bladder margins almost completely across the serosal surface of the amniochorion graft (Figure 14) except for a 1/2 x 1cm area in the centre. The luminal side of the graft was usually encrusted at this stage and in the multilayered groups was often partially extruded into the bladder cavity.

b. Rate of Regeneration

Trends in rates of healing and regeneration are noted in Table XVIII. There were no pathological differences between any of the bladders from the experimental groups at post-operative intervals of 4 weeks or less. Connective tissue regeneration across the peritoneal side of the graft was completed first and maintained the integrity of the bladder during the early healing phase. Urothelium usually had not regenerated yet, amniochorion membranes and remnants were still apparent and inflammatory infiltrates of lymphocytes and macrophages were obvious in all sections. In contrast, biopsies from the control groups contained a normal quantity of urothelium as depicted in Figure 16. None of the biopsies from the experimental groups (Figure 17) displayed a normal amount of collagen, rather granulation tissue and loose collagen were abundant compared to controls. Smooth muscle cells and bundles were not observed during the first 4 weeks in any of the biopsies from the experimental groups but were apparent in the control bladders (Figure 18).

There were differences between biopsies obtained post-operatively at 5-7 weeks compared to those at 4 weeks. Urothelium had regenerated completely in some bladders repaired with 1 or 2 layers of amniochorion and the grafts were no longer recognizable as amniochorion but necrotic debris was seen (Figure 16). Inflammatory infiltrate, collagen and granulation tissue were still abundant as depicted graphically in Figure 17 but by 5 - 7 weeks, scattered smooth muscle cells were present in all sections as demonstrated in Figure 18 although the quantity was much less than
that of controls.

Biopsies obtained at post-operative intervals of 8 weeks or longer showed differences in rates of regeneration between the experimental groups. Urothelial regeneration was complete in all bladders repaired with a single-layered graft but complete in 80% of the double-layer and 75% of the quadruple-layer repair groups (Figure 16). Necrotic debris was no longer obvious in single and double-layer groups but was present in 50% of the quadruple-layer group and inflammatory cells were less abundant in all groups compared to post-operative intervals less than 8 weeks. There was still an abnormal amount of collagen in biopsies from bladders from experimental groups (Figure 17). Smooth muscle cells and bundles were noted in all single and double-layer groups but only in 75% of the quadruple-layer group (Figure 18), although the quantities of cells and bundles in the latter group were not necessarily less than those noted in the other two, as will be reported in the next section.
Figure 14. Diagram of bladder during regeneration, 3 weeks post-operatively, revealed connective tissue overgrowth on the outer surface of the graft, meanwhile the luminal surface had become encrusted.
Figure 15. Biopsy at 3 weeks post-op. Granulation tissue (Gt) has extended across the double-layered amnionchorion graft (AC) on the peritoneal surface and appears to originate from the bladder margin (Bm). One area shows evidence of organization. Fibroblasts, macrophages and necrotic tissue are present.
Table XVIII. A Comparison of Bladder Pathology Between the Control and Experimental Groups at Varying Intervals Post-operatively.

<table>
<thead>
<tr>
<th>Group: Post-op interval</th>
<th>Urothelium</th>
<th>AC ND</th>
<th>L/I</th>
<th>C/GT</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at 4 wks; n=2</td>
<td>2/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>2/2</td>
</tr>
<tr>
<td>at 11 wks; n=3</td>
<td>3/3</td>
<td>0/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Single layer:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4 wks; n=2</td>
<td>0/2</td>
<td>(AC)2/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>5-7 wks; n=2</td>
<td>2/2</td>
<td>(ND)2/2</td>
<td>0/2</td>
<td>0/2</td>
<td>2/2</td>
</tr>
<tr>
<td>&gt;8 wks; n=6</td>
<td>6/6</td>
<td>0/6</td>
<td>1/6</td>
<td>0/6</td>
<td>6/6</td>
</tr>
<tr>
<td>Double layer:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4 wks; n=3</td>
<td>0/3</td>
<td>(AC)3/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>5-7 wks; n=3</td>
<td>3/3</td>
<td>(ND)3/3</td>
<td>0/3</td>
<td>0/3</td>
<td>3/3</td>
</tr>
<tr>
<td>&gt;8 wks; n=5</td>
<td>4/5</td>
<td>0/5</td>
<td>2/5</td>
<td>0/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Quadruple layer:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4 wks; n=4</td>
<td>0/4</td>
<td>(AC)4/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>&gt;8 wks; n=8</td>
<td>6/8</td>
<td>(ND)4/8</td>
<td>4/8</td>
<td>0/8</td>
<td>6/8</td>
</tr>
</tbody>
</table>

Urothelium, normal quantity; AC, presence of amniochorion; ND, presence of necrotic debris; L/I, normal quantity of lymphocytes and inflammatory cells; C/GT, normal quantity of collagen; SM, presence of smooth muscle regeneration; wks, weeks post-operatively; n, sample size.
Figure 16. The percentage of bladders with a normal quantity of urothelium after various post-operative intervals. Zero percent indicates there were no bladders with normal urothelium in some groups.
Figure 17. The percentage of bladders with a normal quantity of collagen after various post-operative intervals. Zero percent indicates there were no bladders with the normal quantity of collagen in the experimental groups.
Figure 18. The percentage of bladders containing smooth muscle cells and bundles. Zero percent represents the absence of any smooth muscle cells or bundles.
c. Histological Features of Regeneration

The sequence of events observed during healing and regeneration is illustrated in Figure 19. In all experimental groups, amniochorion elicited an immediate response as evidenced by the influx of lymphocytes, polymorphonuclear cells, and macrophages. Despite the fact that amniochorion was still identifiable after 3 to 4 weeks, it was undergoing degradation (Figure 20). The amniotic epithelium lacked the distinct cells and nuclei seen in normal amniochorion (Figure 21), the cytoplasm was vacuolated and granular and there were foci of debris. The mesenchymal layer of chorion was infiltrated with lymphocytes, macrophages and polymorphonuclear cells and cells comprising the chorion were vacuolated and granular. Resorption of the graft took weeks to complete as necrotic remnants were still apparent after 9 weeks. The bladder-graft junction was obvious as the 2 tissues were still histologically distinct. The bladder adjacent to the graft was intensely inflamed though and contained lymphocytes and macrophages.

Five to seven weeks after reconstruction, the amniochorion graft was no longer apparent microscopically in single and double repair groups. There were focal areas of necrotic debris surrounded by lymphocytes, macrophages and occasional polymorphonuclear leucocytes. In the single-layer group urothelium had regenerated across the defect (Figure 22), but urothelial overgrowth in the double-layer group was slight after 5 weeks and was not substantial until after 7 weeks (Figure 23). Granulation tissue and loose and dense collagen were abundant and formed the major portion of the bladder wall in both single and double-layer groups (Figure 24), and fibroblasts and myofibroblasts were numerous. There was some evidence of smooth muscle regeneration in both groups, as occasional smooth muscle cells and organized bundles bridged the junction and extended into the defect (Figure 25). The origin of these smooth muscle cells appeared to be muscle from adjacent normal bladder wall, however, isolated smooth muscle cells were also present within granulation tissue in the subepithelial region and were usually adjacent to capillaries as seen in Figure 26.
FIGURE 19. A hypothetical sequence of events during graft degradation and subsequent bladder wall reconstitution during the 4 - 17 week post-operative follow-up.
Biopsies obtained at post-surgical intervals of eight weeks or longer from the single layer group, revealed that urothelium had regenerated completely and had generally attained normal thickness although greater folding was present within the grafted portion - possibly a hyperplastic effect. One biopsy from the double- and two biopsies from the quadruple-layer groups lacked urothelial regeneration at the centre of the defect (Figures 27, 28) but biopsies near the margins contained normal urothelium so with time these cells may have covered the entire reconstructed area. Occasional foci of necrotic debris were still present in 50% of the quadruple-layer repairs (Figure 27) but single and double-layer repaired bladders lacked any evidence of graft material. Macrophages and lymphocytes were still apparent in biopsies from all experimental groups although the quantity appeared somewhat reduced from that seen at earlier post-operative intervals. Subepithelial granulation tissue and loose and dense collagen throughout the bladder wall continued to predominate regardless of the type of repair. Smooth muscle cells and bundles appeared to be greater after 8 weeks in all groups. While all bladders repaired with single or double-layer grafts contained smooth muscle cells, two bladders from the quadruple-layer group appeared to lack smooth muscle. Despite this lack, biopsies from two of the quadruple-repaired bladders, which were positive for smooth muscle, contained the greatest quantity of smooth muscle seen in all sections regardless of the type of repair (Figure 29). Again, smooth muscle cells appeared to infiltrate the graft from adjacent normal tissue, but as well, muscle cells and bundles were present as islands within the granulation tissue (Figure 30).
FIGURE 20. Amniochorion graft (2 weeks post-operative) stained with Masson's (25x). The membranes were still apparent, however cells lacked distinction and many were vacuolated and granular. Necrotic debris was present.
FIGURE 22. Single-layer reconstruction. Bladder section (6 weeks post-operative) stained with Masson's trichrome (96x). There is evidence of urothelial regeneration (U) and smooth muscle bundles (Sm).
FIGURE 23. Double-layer reconstruction. Bladder section (5 weeks post-operative) stained with Masson's stain (25x). Urothelium (U) was scant but did show signs of regeneration. Granulation tissue (Gt) was extensive and smooth muscle cells (Sm) were seen extending into the graft area.
FIGURE 24. Double-layer reconstruction. Bladder section (7 weeks post-operative) stained with Masson's (96x). Lower right corner contains an aggregation of macrophages (M). Fibroblasts and myofibroblasts are abundant (*).
FIGURE 25. Tapering cell process of a myofibroblast containing abundant cytofilaments and dense bodies (>). The nucleus is indented and the rough endoplasmic reticulum scant. (5470X).
FIGURE 26. Double-layer reconstruction. Bladder section (11 weeks post-operative) stained with Masson's (25x). Urothelium (U) appeared normal. Granulation tissue (Gt) and collagen (C) predominated, however, some smooth muscle cells (Sm) appeared at the wound margin and some within the granulation tissue.
FIGURE 27. Double-layer reconstruction. High power view (96x) of Figure 26 showing smooth muscle cells (Sm) and bundles within granulation tissue (Gt).
FIGURE 28. Quadruple-layer reconstruction. Bladder section (9 weeks post-operative) stained with Masson's (25X). Necrotic debris was still present within the regenerated bladder wall after 9 weeks.
FIGURE 29. Quadruple-layer reconstruction. Bladder section (16 weeks post-operative) was stained with Masson’s (25X). Urothelium (U) was thin or nonexistent. Granulation tissue (Gt) and collagen (C) predominated, while smooth muscle (Sm) regeneration was limited to small bundles.
FIGURE 30. Quadruple-layer reconstruction. Bladder section (17 weeks post-operative) was stained with Masson’s (25X). Urothelium (U) and smooth muscle (Sm) has regenerated. Necrotic debris (*) was still present.
FIGURE 31. Quadruple-layer reconstruction. Bladder section (16 weeks post-operative) was stained with Masson's (96X). Isolated smooth muscle cells (Sm) and bundles were present in granulation tissue (Gt).
4. COMPLICATIONS

There were no operative complications nor mortalities in either the preliminary or the control groups. Complications such as leakage from the suture tract, infection and stone formation did occur however, in the experimental groups (Table XIX).

The surgical techniques used to attach the small amniochorion graft to the bladder were unsuitable for attaching a larger single-layer graft. The tension, which was greater on the more extensive suture line, caused tears in the graft at the point of anastomosis resulting in urinary leakage into the abdominal cavity. This problem was rectified by using multiple layers of amniochorion and by attaching the graft into place with a double row of suture such that the second row buried the first row.

Another problem encountered early in this study was infection of the bladder with Streptococcus faecalis which was introduced during catheterization procedures. Rabbits were successfully treated with antimicrobial therapy and to prevent further problems of infection, their urine was monitored by routine culture.110

Stone formation, probably due to crystallization of urinary precipitates on necrotic graft debris, occurred in 44% of the animals with single-layer grafts and in 90% and 87% of those with double- or quadruple-layer grafts (Figure 32). Forty percent of the stones occurred at 2-4 weeks while 60% of the stones occurred between 5-9 weeks. The stones were suspended from the graft and consisted of portions of the luminal layers of the amniochorion graft. Encrustation of the graft rather than stone formation was noted after 4 weeks in some cases. Chemical analysis of stones from infected bladders revealed magnesium ammonium phosphate, calcium, and carbonate, however, they did not reveal microbial infection when cultured. Stones from noninfected bladders were composed of carbonate and phosphate. In either case, stones failed to recur once they were removed (Figure 33).
FIGURE 32. Radiograph of bladder from Rabbit #1135 at 5 weeks. A large stone is apparent and metallic clips approximate the graft area.
FIGURE 33. Radiograph of bladder from rabbit #1135. Bladder configuration and capacity were normal after 16 weeks. There was no recurrence of stone formation nor of leakage.
TABLE XIX. Complications Encountered in the Experimental Groups: Single, Double and Quadruple Layers.

<table>
<thead>
<tr>
<th>Complication</th>
<th>Single Layer</th>
<th>Double Layer</th>
<th>Quadruple Layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stones or encrustation</td>
<td>44%</td>
<td>90%</td>
<td>87%</td>
</tr>
<tr>
<td>Extravasation of urine</td>
<td>18%</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Bladder infection</td>
<td>18%</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Respiratory infection</td>
<td>12%</td>
<td>nil</td>
<td>nil</td>
</tr>
</tbody>
</table>
Part Five: DISCUSSION
CAPACITY

Bladder capacities of rabbits were affected by surgery whether bladders were reconstructed with amniochorion, bladder dome or simply resected and closed. Pre-operative capacities in this study were 16 ml - 121 ml (mean 50) while in another study, capacities in hypnotized rabbits were 60 - 90 ml (mean 63). 

In control group A, in which the bladder dome was transected then reattached, there was a nonsignificant trend to decreased capacity after 4 weeks post-operatively but by 11-12 weeks capacities had increased and were not significantly different from pre-operative values. A similar operation is done in humans to relieve urgency and pain and these patients usually have bladder irritability and increased frequency of urination for 1-3 weeks after surgery. There is no tissue loss, so contraction of the denervated bladder base and edema may decrease capacity and temporarily increase frequency as normal capacity usually returns once irritability has subsided. Bladder capacity in control rabbits in the present study may decrease for similar reasons. However, the bladder was transected above the base to avoid the richly innervated detrusor area, so inflamed muscle, which is stiffer and less able to stretch, and edema may have decreased capacity. The other group of controls, in which bladders were resected then closed without additional tissue, had significantly decreased capacities after 11-12 weeks. While capacities were not measured volumetrically after 4 weeks in two animals the bladders appeared larger after 11-12 weeks compared to 4 weeks. Studies in the literature have shown that partially resected bladders were able to regain a portion of their original size after 12-18 months so a longer follow-up may have revealed increased capacities. The mechanisms may have included regeneration and hypertrophy and mechanical distention (viscoelasticity).

Viscoelasticity is due to the behaviour of long-chain macro-molecules when exposed to stress. A viscoelastic structure has two properties: 1) Creep - if stress (force) is maintained then strain (elongation) increases with time; 2) Stress-relaxation - the stress decreases with time. These molecules slide relative to one another with time and eventually rupture at
points of interconnection, with consequent creep and stress-relaxation behaviour. This known property of bladder tissue may have been partially responsible for the gradually increasing post-operative capacities in the control rabbits.

Bladder capacities in rabbits of the single and quadruple-layer groups were significantly decreased at 4 weeks or less post-operatively and there was a nonsignificant trend to smaller capacity in the double-layer group according to a paired t test. Fishman et al\textsuperscript{14} also assessed amniochorion as a graft in rabbit bladder reconstruction and found that post-operative capacities, measured in anaesthetized animals by cystograms rather that volumetric studies were not different from pre-operative. They did not state the amount of contrast material injected nor the flow rate. Other investigators suggest that animals be conscious because reflexes may be blocked by anaesthesia. Bladder capacities of hypnotized rabbits were 60 - 90 ml which increased to 70 - 120 ml when the same rabbits were anaesthetized.\textsuperscript{105} A physiologic flow rate to fill the bladder and instillation fluid at body temperature are also recommended.

All experimental groups had increased bladder capacities after 5 to 17 weeks compared to 4 weeks or less post-operative. A comparison of mean percent changes between intervals less than 4 weeks and greater than 5 weeks in the single layer group were: -46% and -14%; in the double-layer group: -58% and +32%; and in the quadruple-layer group: -61% and -32%. There was a general trend of increasing capacity with time. An analysis of variance comparing the number of layers used in the reconstruction regardless of the post-operative interval, suggested that capacities of the quadruple-layer group were significantly different from single- and double-layer groups. During the four week follow-up this was probably the result of greater bulkiness of the quadruple-layer grafts. However at intervals longer than 5 weeks a prolonged chronic inflammatory response may have been necessary to degrade and remove the greater quantity of tissue. When groups were analysed for time interval only, regardless of the number of layers, capacities were significantly reduced at 4 weeks or less. An analysis of variance of groups assessed according to both the number of layers and time revealed no significant differences
between groups at similar post-operative intervals.

In all groups, decreased capacities, (at 4 weeks or less) could have resulted from: stones, failure to reconstruct with the same amount of tissue as removed, decreased viscoelasticity due to increased collagen, contraction of collagen during healing, trauma of surgery or a combination of these. Stone formation may have contributed to the decreased capacities but may not have been a major factor because 60% of stones were discovered between 5 and 9 weeks and while bladder capacities at this time were decreased they were no longer significantly different from pre-operative.

Reconstruction with less tissue than was resected was avoided by the surgical technique: 1) stay sutures were placed on the circumference and anchored under tension to maintain the defect size; and 2) an inflated foley balloon was placed within the defect before the graft was sutured.

Collagen was increased, compared to normal bladder, in every histological section viewed and was obvious by 4 weeks when smooth muscle was just beginning to appear. Collagen is stiffer than muscle and hardly shows the stress-relaxation of muscle during bladder filling and may explain why these somewhat fibrotic bladders tended to have reduced capacities.

The contraction of granulation tissue and collagen, a phenomenon seen in scar tissue, may have reduced the early post-operative capacity in the experimental rabbits. After 6 weeks smooth muscle cells began to organize into small bundles which became more numerous over the next 10 weeks. Despite abundant collagen, smooth muscle restoration may have contributed to a gradually expanding bladder capacity and incomplete contracture.

Traumatized bladder wall may remain contracted for several days or weeks following surgery or electrocoagulation. Even control rabbits had decreased capacities during the early post-operative phase. Generally, in this study capacities were similar at identical post-operative intervals, whether bladders were reconstructed with 1, 2 or 4 layers of amniochorion.
Some post-operative capacities in the single- and double-layer groups were actually greater than pre-operative capacities and may be attributed to larger grafts as the surgical technique was devised to ensure a maximum size.

**IMMUNOLOGIC RESPONSE**

The immunogenicity of human amniochorion grafted into rabbit bladder has not been previously studied in detail. In the present study, it was assessed by observation of graft degradation, and detection of humoral anti-human antibodies.

Immunological and histopathological studies showed that human amniochorion underwent degradation when used in reconstructive bladder surgery. Cryostat sections of amniochorion-grafted bladders, during the first nine weeks post-operatively, fluoresced when treated with mouse anti-human HLA-A,B,C, antibodies and goat anti-mouse IgG conjugated to FITC. More fluorescent cells were detected during the first four weeks, then the numbers decreased as degradation of amniochorion proceeded. Sections from controls and bladder tissue cryopreserved at surgery were negative for the same panel of anti-human HLA antibodies. HLA-A,B,C antigens are present on chorion and maternal decidual cells, but scarce on amnion, so persistence of the amnion portion may go undetected. Histopathological examination showed amniochorion degeneration. After 3 weeks bladder walls were intensely inflamed (polymorphonuclear leucocytes, mast cells, macrophages and lymphocytes were numerous) and cells within the graft were vacuolated and necrotic. By 9 weeks the grafts were not apparent but foci of necrotic debris and inflammatory cells were seen. Inflammation was present in most preparations and may have been generated in response to the xenograft, ischaemic injury, and surgical trauma. The grafts were not vascularized and tissues undergoing ischaemic necrosis are often infiltrated with polymorphonuclear leukocytes (PMN's) and macrophages. These cells, in addition to lymphocytes are evident following surgical trauma as well. Therefore the
inflammatory cascades generated in response to tissue injury, ultimately resulted in graft degradation and resorption during the 16 week follow-up.

Antihuman antibodies detected in sera from experimental rabbits were cytotoxic to human lymphocytes in a complement-dependent cytotoxicity assay (CDC). The antibodies recognized antigens which may belong to Class I and/or trophoblast-lymphocyte crossreactive (TLX) antigen systems. These antigens have been reported by others investigating the immunogenicity of pellets of human trophoblast implanted into rabbits. In the present study strongest responses were recorded from sera collected between 2 and 9 weeks but not all rabbits produced strong positive responses to all members of the lymphocyte panel. The lack of response to certain panel members may indicate allotypic differences between antigens on the amniochorion graft and a member’s lymphocytes, in which case, the antihuman antibodies do not recognize the antigens associated with those particular lymphocytes. Such polymorphism for the Class I and TLX antigen systems have been well documented in the literature.

To determine if the antihuman antibodies were specific for Class I histocompatibility antigens, sera were absorbed with platelets. Platelet-absorbed sera, from ten of fifteen experimental rabbits, contained antibodies cytotoxic to human lymphocytes in a CDC assay. Antigens eliciting this response may have been trophoblast-lymphocyte crossreactive antigens (TLX). Such an antigen was identified by others using a monoclonal antibody originally raised to an antigenic determinant of Class I HLA marker on lymphocytes which happened to have a domain with similarities to a trophoblast antigen. The positive responses in this study may also, however, have resulted from allotypic differences between the pooled platelets used in the absorption protocol and the grafted amniochorion, in which case platelet-absorption would not be effective in removing platelet-associated antigens such as Class I transplantation antigens. Platelet-absorbed sera which failed to react in the CDC assay did react positively when the sera were assayed for CDC prior to platelet-absorptions and following amniochorion absorptions.
Thus platelet-absorption in these cases removed antibodies cytotoxic to lymphocytes, (likely Class I anti-HLA-A,B,C, antibodies).

Cryostat sections of human amniochorion were reacted with platelet-absorbed rabbit sera and anti-rabbit IgG conjugated to FITC. A strong fluorescent response was observed in all sections tested from the experimental groups. This may indicate that rabbits also produced antibodies to human antigens present in the amniochorion which were not removed in the platelet-absorption procedure and were not HLA antigens (perhaps TLX and other amniochorion-associated antigens). The positive response may also have resulted from an ineffective platelet-absorption due to allotypy between the platelets and the amniochorion (as discussed before for the CDC results) because the CDC response following platelet-absorption was positive in some sera.

To determine if antibodies were generated against amniochorion-specific antigens, sera were absorbed with amniochorion and tested with the CDC assay and with indirect immunofluorescence. Sera produced strong positive responses in the CDC assay, suggesting the presence of non-trophoblastic antibodies which were cytotoxic to human lymphocytes. These antibodies were probably directed towards Class I antigens. Poor expression, and/or low density of Class I antigens within the amniochorion would prevent complete removal of antibodies to such antigens during absorption procedures. Chorion, which is comprised of cytotrophoblast and which expresses HLA antigens has been noted to elicit a rejection response when left on a wound for several days. In another study, chorion auto- and allo-grafts were rejected within 14 days. Rabbits in the current study probably generated antibodies against HLA Class I antigens on the chorion and maternal cells, in which case, this immunologic response may have played a role in graft rejection and degradation. Amnion appeared to lack or poorly express HLA Class I transplantation antigens as investigators failed to detect these antigens using monoclonal antibodies. SDS polyacrylamide gel electrophoresis (Western Blot) of amniotic epithelium showed only faint bands corresponding to the 43,000 MW HLA-A,B,C chain when gels were reacted with monoclonal antibodies. Despite the presence of Class I
antigens on the cytotrophoblast and the maternal decidual cells of the chorion\textsuperscript{102,103} the quantity may have been insufficient to completely remove Class I antibodies. As mentioned previously, the five rabbits which produced weak or negative responses in the CDC test following platelet-absorption did react strongly in this test indicating that Class I antibodies were present and were not removed by amniochorion absorption.

Cryostat sections of amniochorion failed to fluoresce (or very weakly fluoresced) when reacted with amniochorion-absorbed sera and goat antirabbit IgG conjugated to FITC. Specificity of the antisera for amniochorion is therefore suggested because its reactivity was completely removed by absorption with amniochorion but not with platelets. Amniotic epithelium antigens\textsuperscript{101}, amniotic basement membrane antigens (AA\textsubscript{1}, AA\textsubscript{2}, AA\textsubscript{3})\textsuperscript{84,102,117} and trophoblast antigens (TA\textsubscript{1}, TA\textsubscript{2})\textsuperscript{117} have been identified by others using immunofluorescence. In these studies antitrophoblast sera reacted positively with trophoblast but not with any other tissues. Absorption with amnion did not change the pattern of immunofluorescence but absorption with chorion removed reactivity. Rabbits in this study therefore, may have produced antibodies to amnion and trophoblast antigens, but whether or not these antibodies played a role in graft rejection was not determined.

The immunological techniques used in this investigation provided indirect evidence for HLA Class I, amniochorion and TLX specificity. Immunological results observed in our animal model may not be extrapolated to the use of human amniochorion in humans. More sensitive and sophisticated methods used by other investigators\textsuperscript{101-103} provided direct evidence for Class I and amniochorion-associated antigens but this was not the objective of this research. Identification of these antigens may be pertinent if clinical bladder reconstruction using amniochorion is considered. Amnion may be less immunogenic than chorion which contains Class I transplantation antigens and which elicits a rejection response when left on wounds for several days.\textsuperscript{83,90} Others have studied the immunogenicity of human amnion as an allograft implanted subcutaneously into the arms of volunteers.\textsuperscript{97} Paraffin-embedded sections revealed
granulation tissue, and infiltration of lymphocytes, macrophages, plasma cells and eosinophils. The volunteers did not produce anti-HLA antibodies and the immune response to the graft was low grade and chronic as the amniotic epithelial cells appeared to survive. Amnion, placed on wounds as a biological dressing is not acutely rejected but instead is gradually degraded as host tissues regenerate. Further animal studies using allografts of amniochorion or multi-layered amnion to reconstruct bladders are required to determine the extent of the immune response and whether or not it affects regeneration of bladder wall.

BLADDER REGENERATION AND REPAIR

Tissue repair within control bladders was completed by typical wound healing mechanisms. The xenograft in the experimental group however, presented an additional challenge to the host, resulting in a chronic inflammatory response and a prolonged healing episode.

Bladder wounds in control groups were not macroscopically obvious after 3 weeks. Microscopic evaluation showed that urothelium had regenerated across the incision, probably by replicating reserve urothelial cells. Granulation tissue was still apparent and smooth muscle bridged the defect. Urothelium and submucosa were normal, but the smooth muscle layer was thinner and the amount of connective tissue was thicker than that seen in normal bladder. Similar results were described by others studying bladder regeneration following resection.

Control bladders, resected then closed without tissue replacement, increased slightly in size with no apparent distortion of the bladder nor of the upper urinary tract during the 12 week follow-up. Some investigators, however, have reported distortion after several months.

In the present study, multi-layered amniochorion grafts provided a temporary, leakproof
scaffold for regenerating bladder cells. Repair probably resulted primarily from regeneration of cells within the bladder wall but was perhaps hastened by some fibrous contracture of the scar tissue. The xenograft caused an inflammatory response which was more intense and prolonged compared to that induced by surgical trauma in control rabbits. Amnion and chorion, still identifiable after 4 weeks, were obviously undergoing necrosis as dead cells and numerous macrophages, polymorphonuclear cells, mast cells and lymphocytes in the graft and at the bladder margins were observed. Collagen from amnion was occasionally seen after 8 weeks. Other investigators also reported the presence of amnion four weeks after implantation into rabbit bladder\textsuperscript{16} and studies in which amnion was implanted into humans have documented intact amnion as well as amniotic remnants after 3-4 weeks.\textsuperscript{97}

As a consequence of the inflammatory cascades resulting from surgical trauma, ischaemia and immunologic response, many factors interact to initiate the healing process and subsequent tissue replacement. The Hageman factor, present in serum, becomes activated when it contacts collagen, basement membrane and many other substances. In its activated form it triggers the coagulation cascade, the fibrinolytic system and the kinin system, resulting in production of numerous molecules with a variety of effects, some of which are chemotactic to neutrophils and some of which activate the complement cascade thus enhancing the immunologic response and graft degradation. Fibrin, which is produced during the coagulation cascade, extended from the bladder margins to eventually cover the peritoneal aspect of the amniochorion graft in this study. Granulation tissue also proliferated to cover this aspect of the graft and it has been suggested that the fibrin network acts as a framework for advancing fibroblasts and endothelial cells (capillary buds)\textsuperscript{130,131} Thrombin, another component of the blood clotting system, has been shown to have mitogenic and migratory effects on fibroblasts in the human and fibrin-degradation products have stimulatory effects on leukocytes and monocytes.\textsuperscript{132} Fibronectin fragments are chemotactic to fibroblasts and they organize endothelial cells into capillary tubes. Collectively these factors play a role in sealing the defect and in replacing degenerating graft with granulation tissue and eventually, organized connective tissue. The number of fibroblasts and the proportion
of collagen to smooth muscle was greater in the new bladder wall compared to normal bladder.

While granulation tissue was bridging the peritoneal layer of the graft, the luminal layer necrosed and became encrusted with crystalline deposits. Dead cells probably formed a nidus for this crystallization and subsequent stone formation. Rabbit urine contains a high concentration of carbonate and phosphate so a foreign body in such a solution may act as a starting point for precipitation and crystal formation. There was a lower incidence of stones amongst single-layered grafts than multilayered grafts which may reflect a smaller quantity of necrotic tissue to interact with crystals. Encrustation and stone formation on foreign tissue grafts, preserved tissue grafts and synthetic grafts used in bladder reconstruction has often been reported in the literature.

In the experimental groups, regeneration and repair of bladder wall took several weeks. Reconstitution of bladder wall generally took longer in the multilayered groups and may be attributed to slower removal of a greater quantity of tissue. Urothelium on the luminal layer appeared to regenerate at a slower rate than connective tissue on the peritoneal side of the graft (Figure 16). Normal urothelium was seen in all bladders with single-layered grafts and in 4 of 9 multilayered grafts after 5 to 9 weeks. Five of nine bladders repaired with multi-layered grafts followed for a similar period of time however failed to show normal urothelium, being absent in 3 cases, and thin in 2. Histological evaluation of reconstructed bladders followed for 11 to 16 weeks demonstrated fairly normal urothelium. Reports in the literature have documented the regenerative capabilities of urothelium following bladder reconstruction with various synthetic and biological materials. Urothelium failed to regenerate when intact bowel, polyvinyl sponge, silastic grafts and velour-nylon-silastic grafts were used to reconstruct bladders.

Not all cells within the bladder have the same regenerative capability as urothelium. Bladders biopsied after 4 weeks did not contain identifiable smooth muscle cells, but after 5 to 9 weeks
infiltration of smooth muscle cells from defect margins was observed. Sections from single-layered grafts differed from multilayered grafts in that smooth muscle cells were more numerous and more often organized into small bundles. Biopsies obtained after 11 to 16 weeks revealed smooth muscle cells and bundles in all but 2 specimens regardless of the type of graft. This finding supports the belief that smooth muscle does regenerate in the bladder. Bladders were followed for 17 weeks only, so the full extent of smooth muscle regeneration was not established but longer follow-up may provide this information.

Many studies using tritiated thymidine incorporation and light and electron microscopy have documented smooth muscle regeneration in the bladder and urinary tract but whether or not its proliferation plays a significant role in reconstituting bladder wall has not been determined. Examination of bladders reconstructed with omentum, pericardium, gelatin sponge, preserved bladder grafts, intestinal grafts devoid of mucosa, poly (alpha amino acids) membrane, or amniochorion demonstrated that smooth muscle bridged the defects and proliferated to produce varying quantities of smooth muscle cells and bundles. In these studies, connective tissue initially filled the defects, then smooth muscle cells appeared after 6 weeks. These results confirm those of the present investigation in which smooth muscle cells appeared after 5 weeks then gradually increased. During the 17 week follow-up, however, the normal quantity of smooth muscle had not been attained and collagen still predominated in many cases. The organized muscular component, however, may have enabled bladders to attain adequate post-operative capacities.

The source of regenerating smooth muscle cells in the bladder wall has not been clearly defined in previous studies nor was it determined in the present investigation, but myoblasts from adjacent bladder margins, precursor mesenchymal cells, myofibroblasts or pericytes may have been sources of smooth muscle. Numerous muscle cells extended from normal bladder musculature into the area of repair. This appeared to be the greatest source of cells and others have reported similar results in which myoblasts appeared, on electron microscopy, to migrate
from the wound margins. Tissue injury results in the elaboration of factors which stimulate smooth muscle proliferation (PDGF, platelet factor 4, and products from the lipooxygenase pathway). Macrophages, during inflammation, also secrete growth factors for smooth muscle cells, fibroblasts, and endothelium. In the current study, while migration and/or proliferation of myoblasts from the wound margin may have accounted for most of the smooth muscle regeneration, the presence of such cells and bundles at sites distant from the margins may indicate another source. Scattered smooth muscle cells and a few organized bundles were observed in areas where the defect was being repaired with granulation tissue. A sufficient number of myoblasts, to allow fusion and muscle formation, may have migrated this distance. However, an alternative hypothesis is that in this area of active cellular proliferation, uncommitted mesenchymal cells may have been recruited into the proliferative pool of cells then differentiated into smooth muscle cells in response to the contractile function of the bladder wall. Two such cells may have been the myofibroblast and/or the pericyte.

Myofibroblasts were apparent after 5 to 9 weeks. They share ultrastructural characteristics with both fibroblasts (rough endoplasmic reticulum) and smooth muscle cells (nuclear indentations, myofilaments, dense bodies and attachment bodies), as seen in Figure 25. The origin of the myofibroblast has not been positively determined but indirect evidence has indicated that it may be the fibroblast, the smooth muscle cell or a primitive mesenchymal cell. A relationship between these three cells, the fibroblast, myofibroblast and the smooth muscle cell appears to exist and all three may actually differentiate from the primitive mesenchymal cell. If this pluripotent mesenchymal stem cells exists and if smooth muscle cells represent an alternative differentiated form of the myofibroblast then the latter cell may have accounted for the presence of smooth muscle cells within the granulation tissue in the current study. Stimulus for this particular direction of differentiation may have been the contractile physiology of the bladder. As was noted earlier in this study, during the first four weeks post-operatively, bladder capacity was decreased possibly because of surgical trauma and irritation. As healing proceeded, the bladder resumed its normal contractile function which perhaps
provided the environment necessary for a phenotypic change of the myofibroblast to the smooth muscle cell. This mechanical change may have accounted for such phenotypic changes proposed by others.\textsuperscript{150} Investigators studying the development of the aortic media, ovarian follicles and atherosclerotic lesions reported similar views that myofibroblasts represent a stage in the differentiation of a primitive mesenchymal cell to a mature myocyte.\textsuperscript{147-149} However, there has been no definitive proof that myofibroblasts differentiate into smooth muscle cells. To add further confusion, myoblasts in culture resemble fibroblasts (but may be distinguished by their synthesis of desmin\textsuperscript{151} and the presence of a cell surface antigen\textsuperscript{152}). Histological studies alone cannot be used to distinguish between fibroblasts, myofibroblasts and immature smooth muscle cells.

In the present study, smooth muscle cells and bundles were observed in granulation tissue, in collagen-dense areas away from the wound margins and in close approximation to capillaries. There is abundant evidence in the literature that pericytes, which lie adjacent to endothelial cells in capillaries, may differentiate into smooth muscle cells when there is a functional demand.\textsuperscript{153-159} The pericyte is transported everywhere by growing capillaries in wound healing and during bone formation and appears to retain the capacity to serve as a precursor to other cells.\textsuperscript{154} Pericytes, in culture and in situ possess muscle and nonmuscle isoactins and may play a contractile role.\textsuperscript{155}

The presence of smooth muscle cells and bundles, in the current study, contrasts results reported by others in which materials such as lyophilized human dura, peritoneum, preserved fascia and silastic grafts, were used to repair bladders. These grafts usually produced nonfunctional, fibrotic, contracted bladders with no evidence of smooth muscle regeneration.

Difficulties in providing a functional bladder replacement prompted this current study. The goal was to test amniochorion as a graft which would serve as a temporary scaffold for regenerating bladder wall components while maintaining continence and capacity. A preliminary study, conducted to determine the effectiveness of a small-patch replacement in the
rabbit bladder, revealed that the graft was not sloughed, and there were no leaks nor other complications. Encouraged by these results single-layer grafts were used to reconstruct rabbit bladders. Early post-operative leakage was overcome by using multilayered grafts, however, stones and encrustation of the graft occurred in most multilayered reconstructions. Stone formation can perhaps be eliminated by diuretic therapy. Amniochorion, while maintaining bladder function, served as a temporary scaffold for connective tissue overgrowth and urothelial and smooth muscle regeneration. Collagen was abundant but smooth muscle regeneration may have prevented complete contracture because capacities gradually increased with time. Studies using amniochorion or just amnion as an allograft are required to determine if fibrous contracture occurs after several months and whether or not stone formation can be avoided.

Improvements in bladder reconstruction have occurred over the last 100 years and despite increased patient survival, the use of bowel grafts still result in long-term complications and poor quality of life. While the results from this study cannot be extrapolated to allografts in the clinical setting amniochorion/amnion may become an alternative material to bowel, if fibrous contracture and stone formation can be avoided.
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