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Experimental Studies on the Role of the Gastrointestinal Microflora in Postsurgical Adhesion Formation

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To all animal participants in medical research

without whom we would know so little.....

Om du tänker för länge på nästa steg,
kommer du att tillbringa livet på ett ben.
Kinesiskt ordspråk

Experimental studies on the role of the gastrointestinal microflora in postsurgical adhesion formation

Introduction:
Adhesions occurring after any kind of surgery is a common phenomenon and cause a great deal of morbidity and mortality, incurring a considerable burden on health care systems. Adhesions are especially prominent after lower abdominal and gynecological procedures (60-90 % of patients after one operation) causing infertility, pain syndromes and bowel obstruction as well as complicating subsequent surgery. Despite many attempts there are still no satisfactory ways or means for prophylaxis or treatment. Part of the difficulties is due to lacking in understanding of the basic pathogenesis behind adhesion formation. Infection is regarded as promoting adhesions. Intra-abdominal antibiotics have been used as an adjunct to fertility surgery. Adhesions are particularly common in the abdominal cavity where the close proximity to the gastrointestinal flora may be of importance.

Rationale and Aims:
In order to study the role of the microbial flora in adhesion formation a number of studies were undertaken.

I. To study the role of the gastrointestinal flora in germfree and conventional rats.
II. To study the role of the flora in the germfree and exgermfree states and to develop an objective scoring scale.
III. To study healing of colonic anastomoses and adhesion formation in vitamin A-deficient germfree and conventional rats.
IV. To study the influence of the gastrointestinal flora and two of its species on adhesion formation around surgical anastomoses.
V. To study whether systemic antibiotics may influence adhesion formation.

Materials and methods:
Adhesions were induced in rats by established methods and scored blindly according to special scoring scales, one of which was developed during the work and compared with two counterparts. Germfree and monocontaminated rats were kept in steel isolators, the microbial status being monitored weekly. Vitamin A deficiency was induced with special diet and retinyl esters in liver tissue were analyzed with high-performance liquid chromatography (HPLC). Anastomotic bursting pressures and hydroxyproline content were measured.

Results:
Germfree rats formed less adhesions than conventional counterparts ($p < 0.01$). By turning germfree animals into conventional ones by establishing an ordinary intestinal flora (ex-germfree) the propensity to form adhesions returned ($p < 0.005$). The new scoring scale was not inferior at detecting differences as compared with two other scales. Vitamin A-deficient rats had lower anastomotic bursting pressures than vitamin A-sufficient rats ($p < 0.0005$) , whereas vitamin A-status had no impact on adhesion formation but the intestinal flora-status had ($p < 0.0005$). Adhesion formation increases the more the flora status approaches the normal state ($p < 0.0001$). Amoxicillin / clavulanic acid treated conventional rats had less intestinal bacteria ($p < 0.05$) and formed less adhesions ($p < 0.05$).

Conclusions:
The bacterial flora of the gastrointestinal canal influence adhesion formation but is not essential for adhesions to develop. Restoration of an ordinary flora restores adhesion forming propensity. The new scoring scale is at least as good as scales compared at detecting differences but has advantage in the form of being objective. Vitamin A is important for healing of colonic anastomoses but did not affect adhesion formation whereas the intestinal flora status did, indicating that the mechanisms might be different. The more normal the flora gets the more normal the adhesive response, indicating that different species of bacteria have different adhesiogenic ability. Antibiotics lowering bacterial numbers of the gastrointestinal flora reduce adhesions, but resistance problems may theoretically induce growth of potent adhesiogens.The present findings might help to explain why measures aimed at reducing fibrin do not work on ischemic bowel and around anastomoses and warrant more research, in which germfree animals could be valuable as a model void of the intestinal flora influence.

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List of publications

This thesis is based on the following publications, which will be referred to by their Roman numerals.

I. Bothin C, Midtvedt T. The Role of the Gastrointestinal Micro Flora in Postsurgical Adhesion Formation - a Study in Germfree Rats.
Eur Surg Res 1992; 24: 309-312 (Karger, Basel. www.karger.com).

II. Bothin C, Okada M, Midtvedt T. Experimental Postsurgical Adhesion Formation in Germfree and Exgermfree rats - a Study Using Three Scoring Scales.
J Invest Surg 1999;12:147-150 (Taylor & Francis Limited. www.tandf.co.uk).

III. Okada M, Bothin C, Blomhoff R, Kanazawa K, Midtvedt T. Vitamin A Deficiency Impairs Colonic Healing but not Adhesion Formation in Germ-Free and Conventional Rats.
J Invest Surg 1999;12:319-325 (Taylor & Francis Limited. www.tandf.co.uk).

IV. Bothin C, Okada M, Midtvedt T, Perbeck L. The Intestinal Flora Influences Adhesion Formation Around Surgical Anastomoses.
Br J Surg 2001;88:143-145 (Blackwell Publishing. www.blackwell-synergy.com).

V. Bothin C, Perbeck L, Midtvedt T. Antibiotic Treatment Can Decrease Intra-Abdominal Adhesion Formation.
Submitted.

Abbreviations

- A	vitamin A - deficient
+ A	vitamin A - sufficient
CV	conventional (rats)
EC	E. coli
GF	germfree (rats)
L	Lactobacilli
MAS	macromorphological adhesion grading scale
PAA	plasminogen activator activity
XGF	ex-germfree (rats)

Preface

The studies in this thesis have been performed on comparatively small samples mainly due to the heavy cost of gnotobiotically reared specimens,
and to a minimal extent due to the relatively wearying effect of performing the minute experimental procedures in the closed compartments of the isolators.

¨Failure to reject H_0 *does not imply* that H_0 may be accepted and that there are no differences between the groups. When the sample sizes are small, only relatively large differences are detected by our statistical procedures which lead to rejection of H_0. This is because when the sample size is small and H_0 is in fact true, the probability of large variation in outcomes is also large. As a consequence, it is difficult to distinguish between outcomes reflecting merely chance deviations (when H_0 is true) and true differences
(when H_1 is true). If H_0 is not rejected, then there in fact may be no differences between the groups - or the sample sizes may be so small and / or the variability in the sample so large and / or the differences so small that true differences can not be detected. Before *accepting* H_0 in such cases the researcher should seek corroborating evidence or obtain additional data. As a final note, this caution does not imply that we should not have confidence in the differences between groups if we are able to reject H_0 at a given significance level. These arguments apply with equal force to both parametric and nonparametric tests.¨ (Siegel and Castellan, Jr. 1988)

Background

In 1872 Bryant reported a case of fatal intestinal obstruction due to intra-abdominal adhesions appearing after removal of an ovarian cyst (Bryant 1872).
Since then many attempts to counteract adhesions by various means have been made and much knowledge has been gathered (See reviews: Boys 1942, Krook 1947, Connolly & Smith 1960, Ellis 1971 and 1980, Holtz 1980, Levinson & Swolin 1980, DeCherney 1984, Holtz 1984, Stangel et al 1984, Diamond & DeCherney 1987, Holtz 1990, Jansen 1991, Bothin & Hallberg 1991, Drollette & Badawy 1992, Pados & Devroey 1992, Menzies 1992 and 1993, Stone 1993, Pijlman et al 1994, diZerega 1994, Gomel et al 1996, Risberg 1997, Holmdahl et al 1997, Watson et al 2000, Chegini 2002).
Many methods and substances have initially been reported upon as being successful. However, very few have succeeded to be established in clinical practice due to subsequent equivocal results or side-effects. In fact, reports with conflicting results are numerous in the literature. Nevertheless, today there are a few approved products of the barrier-type (i.e. materials separating surfaces) to be used clinically for adhesion prevention in the abdomen (Farquhar et al 2000, Johns DB et al 2001) but still there is no uniformly effective treatment or adjuvant available (Menzies 1992, diZerega 1994, Gomel et al 1996, Risberg 1997,Treutner & Schumpelick 2000, Liakakos et al 2001). Development is partly hampered by the fact that the pathogenesis of adhesion formation is incompletely understood. The mechanisms underlying the predisposition to form adhesions as well as their site specificity are completely unknown (Chegini 2002).

Magnitude of the problem

Nearly all patients undergoing abdominal surgery develop adhesions (Menzies & Ellis 1990, Ellis 1997). Repeated operations increase the incidence.
Adhesions can cause bowel obstruction, female infertility, abdominal pain (Sulaiman et al 2001) and technical difficulties during subsequent surgery. They may also be routes for spread of gastrointestinal cancer (Triotskii 1967, 1970, Lawrence 1991).
In developed countries adhesions are the most common cause of small bowel obstruction accounting for 70 - 80 % of all cases (Räf 1969, Menzies & Ellis 1990, Ivarsson et al 1997). The life time risk of developing adhesive bowel obstruction after an abdominal operation is estimated to be between 0.3-10.7 %. Up to 1 % of cases occurring during the first year (Menzies & Ellis 1990, Ellis 1998). The cumulative risk of adhesive small-bowel obstruction after (sub)total colectomy is 11 % within one year, increasing to 30 % at ten years (Dijkstra et al 2000). Some

individuals repeatedly develop obstructions.
Estimations of the costs associated with care for adhesions have been performed. In the USA direct costs in 1988 was nearly 1200 million US dollars (Ray et al 1993). A recent investigation in Sweden calculated the direct costs , for care of adhesive bowel obstruction alone during 1992-93, to 100 million SEK (Ivarsson et al 1997).
Roughly 3 % of all surgical admissions are associated with intra-abdominal adhesions (Dijkstra et al 2000).
Apart from the fields of gynecological and abdominal surgery adhesions are encountered and cumbersome in many other areas of surgical procedures, e.g. the ear, eye, pleura, pericardium, tendons, spinal canal and also in peritoneal dialysis.

Past and present theories of pathogenesis

A great deal of interest has been directed towards preventing de-novo adhesions, arising after any surgical procedure, and reforming adhesions, which occur after operations with the objective of dividing adhesions.

There are a vast number of stimuli for adhesion formation such as ischemia, venous stasis, crushing, abrasion, drying, irrigation, foreign materials, blood, infection, endometriosis, chemical and thermal injury (Ellis1962, Swolin 1966, Belzer 1967, Myllärniemi 1967, Ryan et al 1971, Jagelmann & Ellis 1973, Fedor et al 1983, Kaplun et al 1984, O'Leary et al 1987, Kappas et al 1988, O'Leary & Coakley 1992, Sekiba et al 1992)

In the literature theories on the pathogenesis of adhesions have been proposed, fig. 1-3.
A common theme is that peritoneal trauma, ischemia or infection initiate a sequence of events where a central role has been attributed to fibrin.
The hypothesis is that if this fibrin persists beyond the first three days the consequence will be permanent adhesions, but if the fibrin is lysed before the critical time period adhesion-free healing will result.

The classic pathway for adhesion formation was claimed to start with the outpouring of a fibrin-rich intraperitoneal exudate in response to an intra-abdominal insult. Fibrinous adhesions between neighboring viscera appear soon after the insult and in the presence of an intact peritoneum these adhesions are lysed. In the case of damaged peritoneum the adhesions become permanent, fibrous adhesions by vessel and fibroblast invasion. (Menzies 1992), fig. 1.

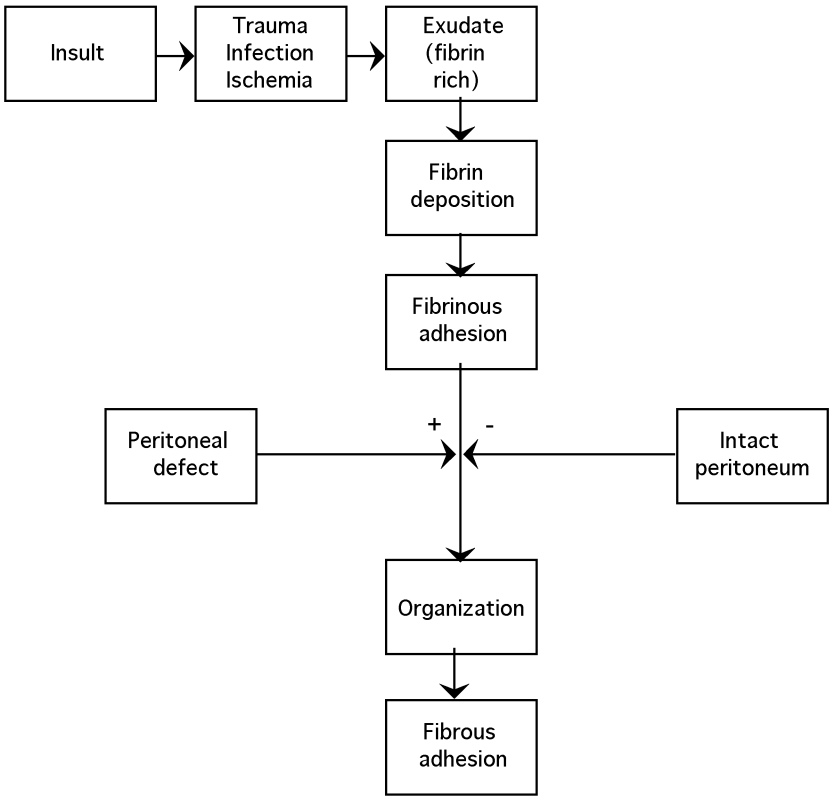


Figure 1. The classic pathway for adhesion formation (Menzies 1992).

In the revised pathway for adhesion formation the lysis of the fibrinous adhesion does not depend on the presence of an intact peritoneum but on the presence of an adequate amount of plasminogen activator activity (PAA). When the activity is reduced sufficiently by trauma, inflammation or ischemia fibrous adhesions will result but when there is only a minor reduction in PAA permanent adhesions will not develop. (Menzies 1992), fig. 2.

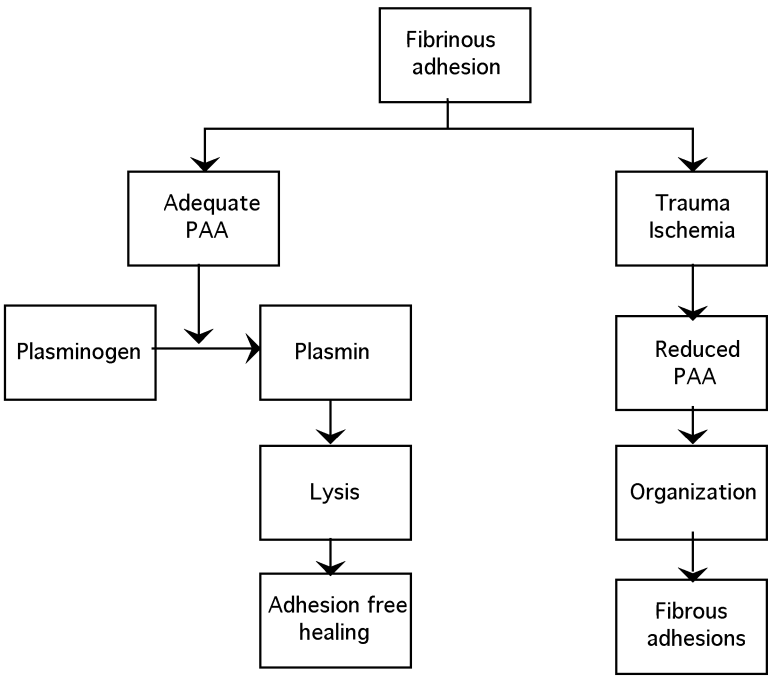


Figure 2. The revised pathway for adhesion formation (Menzies 1992).

In the current concept of the pathogenesis of adhesion formation a more detailed view of the events after peritoneal injury is given, but still with fibrin persistence as the determining event. (Ivarsson 1998, Jack 1998), fig. 3.

In essence, the concept of persisting fibrin is by no means new (Graser 1895, Ladwig 1928, Bogart 1937, Knightly et al 1962) and was questioned rather early (Ryan et al 1971).

Over the last decade advancement in molecular biology has identified many biologically active molecules with the potential of regulating inflammatory and immune responses, angiogenesis and tissue remodeling, events that are central to normal peritoneal wound healing and adhesion formation. Although, insight into their importance in the development of tissue fibrosis has substantially increased, their major roles in peritoneal biological functions and adhesion formation remain at best speculative (Chegini 2002).

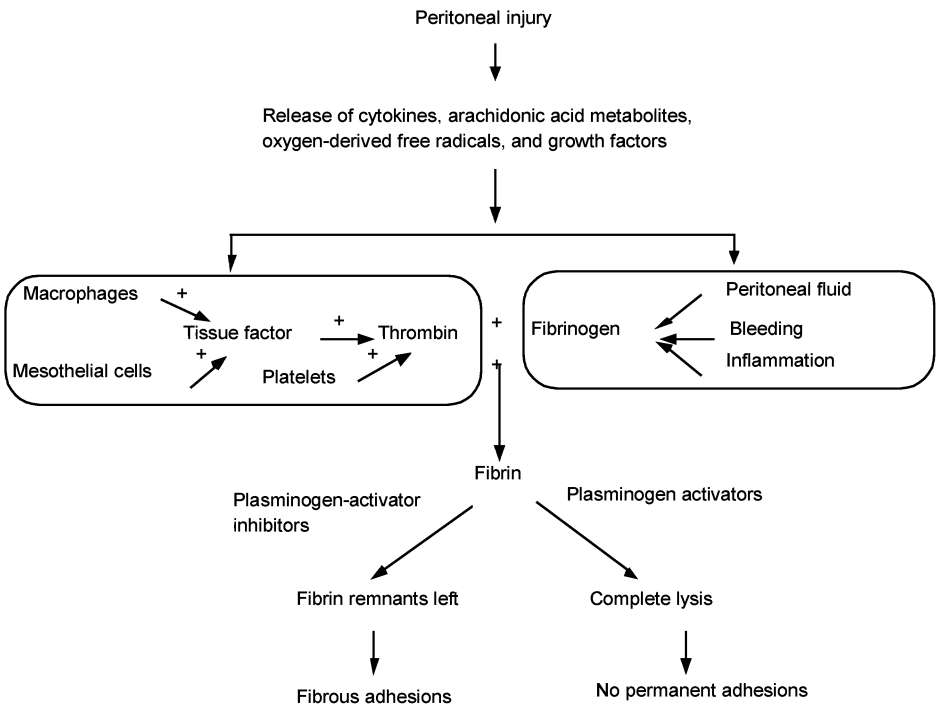


Figure 3. The current concept of the pathogenesis of adhesion formation (Ivarsson 1998, Jack 1998).

Bacteria and adhesions

Early observations as quoted below inspired the studies presented in this thesis.

¨Adhesions require an exudate for their formation, and precipitation of the fibrin in this exudate gives rise to the first agglutination (Graser, 1895, Ladwig, 1928, etc), i.e. a fibrinous, temporary adhesion has formed. An adhesion of this kind will often retrograde, but it may also develop into a permanent adhesion. For this latter process to take place the surface-cells of the peritoneum must be injured (Graser, 1895, Heinz, 1900). An uninjured layer of surface-cells in the serosa is able to prevent formation of permanent adhesions, though only if the underlying tissue is of normal character (Ladwig, 1928). Thus, should the underlying tissue be changed, e.g. through an action from within the intestinal lumen, definite adhesions may form despite an intact layer of surface-cells.¨ (Krook 1947).

”Every abdominal process that provokes an inflammatory state of irritation in the serosa may also be the cause of adhesions (Braun 1924). Among these processes infections are undoubtedly the most common and many animal experiments have been conducted to show the dominant part played by this factor in the formation of adhesions. Guinea-pigs had gauze tampons inserted immediately beneath the peritoneum. One series had sterile tampons, the second had tampons dipped in 5% iodine solution, and a third series had been dipped in a coli-infected solution. The first group had no adhesions, the second group had one animal in ten and in the third group six of ten.
A considerable difference is found in the ability of different infections to produce adhesions. Sometimes after diffuse peritonitis only a few or no adhesions are found, whereas apparently benign infections may give rise to extensive adhesions (Braun 1924 and others). The explanation of this is in part to be found in the different composition given to the peritoneal exudate by different bacteria. Coli pus contains relatively much fibrin and many leukocytes with a tendency to copious adhesion formation as a consequence, while streptococci produce an exudate poorer in fibrin and leukocytes with a lower tendency to agglutinations. As a rule the tendency to adhesion formation diminishes with increasing virulence (Ochsner and Garside 1932, Bogart 1937).” (Krook 1947)

¨An observation of great basic interest has been made by several authors (Pribram 1914, Ochsner and Garside 1932), viz. that a given agent loses its adhesion-preventing effect in the presence of infection even if its prophylactic action has appeared to be convincing in aseptic trials. This is interpreted by Ochsner and Garside to the effect that a bacterial trauma, in contrast to a mechanical one, is continuous, i.e. the trauma still continues to act when the effect of the prophylactic has ceased.¨ (Krook 1947).

The gastrointestinal microflora

The intestinal microflora is a complex ecosystem with hundreds of bacterial species.
Its metabolic functions and interactions with the host are important for health and well being, effects which are difficult to study (von Wright and Salminen 1999). Among the important functions are the synthesis of vitamin K (menaquinones), the production of various nutrients for the mucosa (Bengmark 2000), and to protect against colonization and overgrowth of potentially pathogenic bacteria. The establishment of a normal indigenous microflora is of importance in the development of a normal immune system, and possibly for protection against emerging allergy (Björksten et al 2000, Bottcher et al 2000, Björksten et al 2001, Kalliomaki et al 2001a, Kalliomaki et al 2001b)
Little is known, about the factors that help establish host-microbial symbiosis in the open ecosystem of the intestinal tract, but may involve exchange of biochemical signals between host and symbionts as well as among the bacteria themselves (Hooper et al 1998, Falk et al 1998).

Vitamin A

Vitamin A has many functions, e.g. in vision, immune defence, gene regulation, cell differentiation and morphogenesis. Vitamin A deficiency retards repair in wound healing (Hunt 1986). Retinoids and steroids have antagonistic effects on growth factors and collagen deposition in wound healing, and retinoids can partially reverse corticosteroid-induced impairment of wound healing (Anstead 1998, Wicke et al 2000). Nuclear receptors for retinoic acid have been discovered and additional functions are likely to be found (Harbige 1996, Gerster 1997).

Rationale and aims for the present experiments

Most studies on adhesion formation have been performed in the abdomen in close vicinity to the gastrointestinal tract and its contents.

In order to study the role of the microbial flora in adhesion formation a number of studies were undertaken.

I. To study the role of the gastrointestinal flora in germfree and conventional rats.

II. To study the role of the flora in the germfree and exgermfree states and to develop an objective scoring scale. Scoring scales in use are often crude, involving an element of deciding subjectively which response is the stronger.

III. To study healing of colonic anastomoses and adhesion formation in vitamin A-deficient germfree and conventional rats.

IV. To study the influence of the gastrointestinal flora and two of its species on adhesion formation around surgical anastomoses.

V. To study whether systemic antibiotics may influence adhesion formation.

Materials and methods

Ethics

Applications were submitted to scrutiny by the appropriate ethics committees for the use of laboratory animals and approval was obtained before experiments began.

Animals

AGUS- (Festing 1979) (I, III-V) and DA- rats (Zentralinstitut fur Versuchstierzucht, Hannover, Germany) (II) were used. All animals were allowed to acclimatize for at least 14 days before being subjected to experiments. The animals were maintained on rat chow and tap water ad libitum. Gnotobiotic rats were kept in isolators (Gustafsson1959) and conventional ones in animal rooms with a temperature of 24 ± 2 C°, relative humidity of 55 ± 10 % and a light-dark cycle of twelve hours. The microbial status of the germfree and mono-contaminated rats was checked weekly by cultures of fecal samples.

Anesthesia

Anesthesia was achieved by intramuscular injection in the hind leg of fluanisone and fentanyl (Hypnorm Vet®, Janssen Ph) 0.1 ml / 100 g body weight (I, II) or by intraperitoneal injection of a mixture of equal parts of fentanyl-fluanisone (Hypnorm Vet®) in one part water and midazolam (Dormicum®, Roche, Basel, Switzerland) in one part water (III-V).

Experimental models

The following adhesion inducing methods were used;

caecal crush (Swolin 1966) (I,II,V), achieved by bringing out the caecum, gently milking away the contents before closing a hemostat five times consecutively, beginning at the distal end,

colonic anastomoses (III), constructed by resecting a small part of the colon 2.5 cm proximal to the rectal peritoneal reflection,

colonic and ileal anastomoses (IV), constructed by resecting small parts 5 cm proximal to the ileocaecal junction and 2.5 cm distal to the end of the caecum.

Continuity was restored with eight interrupted 6-0 polypropylene sutures (Prolene®, Ethicon, Norderstedt, Germany).

Vitamin A (III)

Vitamin A-deficiency was induced, after weaning, by feeding for seven weeks with food including vitamin-A (11.400 IU / kg) or not (< 200 IU / kg). Chromatographic separation was used to quantify retinyl esters (vitamin A) in postmortem livers.

Bursting pressure and hydroxyproline content (III)

The colonic segments with the anastomoses in the middle were removed with great care. One end was ligated and at the other end a catheter was secured with silk. The segments were immersed in saline and inflated with air at a pressure increase rate of 2 mm Hg per second. The bursting pressure was defined as the pressure at which air bubbles first appeared.
Subsequently, the anastomotic parts were dissected, freeze-dried, weighed and the hydroxyproline content measured (Prockop & Udenfriend 1960, Juva & Prockop1966).

Observation time

The animals were evaluated after seven days. This period of time, which has been used vastly in the literature, was chosen because the biochemical events deciding whether permanent adhesions will develop are regarded to take place during the first three days and mesothelial healing take five to eight days (Menzies 1992, diZerega 1994).

Scoring adhesions

All scoring was done in a blinded fashion, that is without the investigator knowing to which group a particular animal belonged.
The following three scoring scales were used:

Paper I, II

The macromorphological adhesion grading scale (MAS) (Verrett et al 1989).

0 = no adhesions

1 = local scar formation

2 = adhesive bands

3 = extensive adhesion formation.

Paper II

Adhesion grading scale according to (Nair et al 1974).

0	=	complete absence of adhesions
1	=	a single band of adhesion either between viscera or from one viscus to the abdominal wall
2	=	two bands either in between the viscera or from viscera to abdominal wall
3	=	more than two bands either in between the viscera or viscera to abdominal wall or whole of intestines forming a mass without being adherent to the abdominal wall
4	=	viscera being directly adherent to the abdominal wall irrespective of the number and extent of adhesive bands

Paper II-V

Cumulative adhesion scoring scale (Bot).

Each observation is given one point, the points are added to make up the score. Thus two bands are scored 1 + 1 = 2 points.

+1	No adhesions
+1	Local scar formation
+1	One adhesive band from the omentum to the target organ.
+1	One adhesive band from the omentum to the abdominal scar.
+1	One adhesive band from the omentum to another place.
+1	One adhesive band from the adnexa / epididymal fat bodies to the target organ.
+1	One adhesive band from the adnexa / epididymal fat bodies to the abdominal scar.
+1	One adhesive band from the adnexa / epididymal fat bodies to another place.
+1	Any adhesive band other than described above (e.g. liver to scar).
+1	Target organ adherent to the abdominal wall.
+1	Target organ adherent to bowel.
+1	Target organ adherent to the abdominal scar.
+1	Target organ adherent to the liver or the spleen.
+1	Any other organ adherent.

Scoring scales and statistics

The data obtained by using scales of the above kind is of the ordinal data type. This means that higher values designate ¨more¨ than lower values giving a ranking order of scores, however it says nothing about the distance between values. Calculating means and standard deviations are therefore in error and misleading because the true distances between classes of the scale (i.e. the values obtained) are not equal and is in effect unknown. The median is the descriptive statistic that most appropriately describes the central tendency in this type of data.

Although the scores obtained seem to be discrete the assumption is made that the underlying phenomenon of adhesion formation is a continuous variable.

For the reasons above, non-parametric tests must be used, bearing in mind that these are generally less powerful than parametric tests. (Siegel & Castellan 1988).

The methods employed have been the Fisher exact test, the Kruskal-Wallis-, the Wilcoxon - Mann - Whitney (Mann - Whitney U), the Jonckheere-Terpstra test for ordered alternatives and two-way ANOVA. The level of significance was set at $p < 0.05$.

Results

Paper I

The conventional (CV) rats scored significantly higher than the germfree (GF) ones regarding incidence as well as severity of adhesions. Counting only adhesions engaging the experimental lesion yielded even stronger significance. (Table 1).

Table 1

		Animals with adhesions	MAS scores			
			0	1	2	3
A	(n = 8)	7 (87.5)	1		2	5
B	(n = 10)*	4 (40.0)•	6		1	3
A'	(n = 8)	7 (87.5)	1		2	5
B'	(n = 10)**	2 (20.0)••	8		0	2

Figures in parentheses indicate percentage.

*p = 0.0418 (corrected for ties); **p = 0.0133; •p = 0.057; ••p = 0.0076.

MAS = Macromorphological adhesion grading scale; A = CV controls; B = GF animals; A' = A: there were only adhesions engaging the experimental lesion in this group; B' = B when excluding adhesions not engaging the experimental lesion; one rat in group B had adhesions to the colon several centimeters away from the distal end of the cecum, another rat had one band of adnexal fat to the sutured wound.

Paper II

The GF rats formed adhesions to a significantly lesser extent than their ex-germfree (XGF) counterparts. There were no differences between sexes. All three scoring scales were able to discern statistically significant differences in this study. (Table 2-4).

Table 2 Adhesion scores according to the MAS scale

		0	*1*	*2*	*3*
A (GFF)	n = 10	5		5	
B (GFM)	n = 5	1		3	1
C (GFall)	n = 15	6		8	1
D (XGFF)	n = 12			7	5
E (XGFM)	n = 7			4	3
F (XGFall)	n = 19			11	8

Note. A, germfree females; B, germfree males; C, all germfree; D, ex-germfree females; E, ex-germfree males; F, all ex-germfree. C vs. F, significant at p = .0012

Table 3 Adhesion scores according to the Nair scale

		0	*1*	*2*	*3*	*4*
A (GFF)	n = 10	5	4	1		
B (GFM)	n = 5	1	3	1		
C (GFall)	n = 15	6	7	2		
D (XGFF)	n = 12		1	6	2	3
E (XGFM)	n = 7		1	2	3	1
F (XGFall)	n = 19		2	8	5	4

Note. A, germfree females; B, germfree males; C, all germfree; D, ex-germfree females; E, ex-germfree males; F, all ex-germfree. C vs. F, significant at p = .00001

Table 4 Adhesion scores according to the Bot scale

		1	*2*	*3*	*4*	*5*	*6*
A (GFF)	n = 10	5	4	1			
B (GFM)	n = 5	1	3	1			
C (GFall)	n = 15	6	7	2			
D (XGFF)	n = 12		2	7	3		
E (XGFM)	n = 7		2	3	1		1
F (XGFall)	n = 19		4	10	4		1

Note. A, germfree females; B, germfree males; C, all germfree; D, ex-germfree females; E, ex-germfree males; F, all ex-germfree. C vs. F, significant at p = .00002

Paper III

The concentrations of vitamin A (retinyl esters) in the liver of rats in GF + A and CV + A were 517.0 ± 122.2 and 337.6 ± 63.5 nmol / pg liver tissue, respectively. In GF - A and CV - A no amounts of vitamin A (retinyl esters) could be detected.

There were no significant differences in hydroxyproline HP concentration

In the bursting pressure of colonic segments, - A groups showed significantly lower pressure than + A groups (two-way ANOVA $F = 21.02$, $p < 0.0005$). (Table 5).

In both the cumulative adhesion scoring scale and the anastomosis scoring scale, GF groups had significantly lower scores than CV groups (two-way ANOVA $F = 17.77$, $p < 0.001$; $F = 20.61$, $p < 0.0005$; stratified Mann - Whitney $p < 0.001$, $p < 0.01$; both scores combined two-way ANOVA $F = 23.28$, $p < 0.0005$). (Table 6).

Table 5 Bursting pressure of colonic segments

Group	*Bursting pressure (mm Hg)*	*(Number)*
GF - A	104.0 ± 32.1	(4)[a]
GF + A	158.6 ± 17.5	(7)
CV - A	116.5 ± 3.8	(4)
CV + A	146.5 ± 15.8	(4)[a]

Note. GF - A, germ-free rats not given vitamin A; GF + A, germ-free rats given vitamin A; CV - A , conventional rats not given vitamin A, CV + A, conventional rats given vitamin A.

[a] Two rats in the GF - A group and one in the CV + A group were excluded because the anastomosis leaked.

Table 6 Assessment of adhesion formation

Group	*(Number)*	*Cumulative adhesion scoring scale*	*Anastomosis scoring scale*
GF - A	5[a]	2.20 ± 0.45	2.60 ± 0.89
GF + A	7	2.71 ± 0.76	2.86 ± 1.07
CV - A	4	5.00 ± 1.16	4.50 ± 1.00
CV + A	4[a]	4.75 ± 2.36	5.00 ± 0.82

Note. GF - A, germ-free rats not given vitamin A; GF + A, germ-free rats given vitamin A; CV - A, conventional rats not given vitamin A, CV + A, conventional rats given vitamin A.

[a] One rat in the GF - A group and one in the CV + A group, which developed abscesses, were excluded.

Paper IV

The results are presented in detail in Fig. 4. Horizontal lines depict medians.

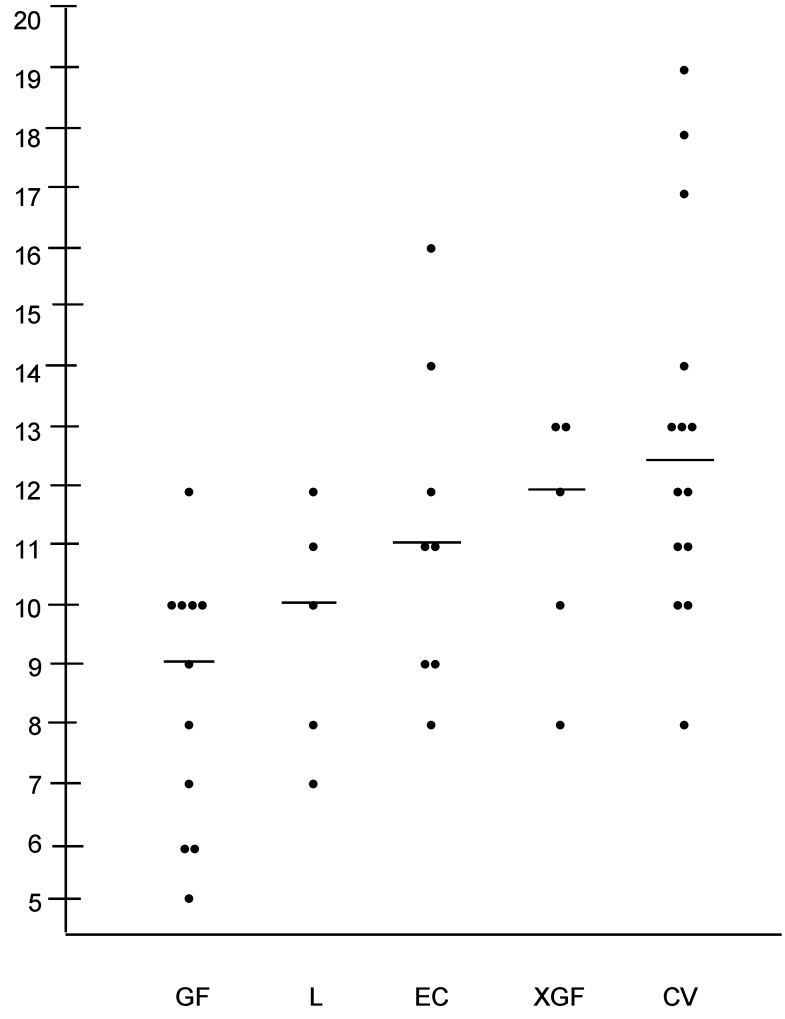
No bacteria other than the presumed species were detected during the study period.

There were no wound infections.

Adhesion formation increases as the flora approaches the normal state

($p < 0.0001$, Jonckheere - Terpstra test).

Fig. 4. Anastomoses adhesion score

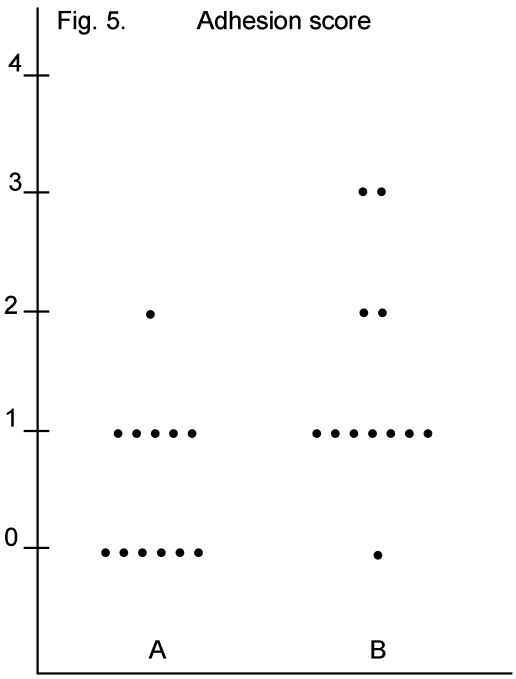


GF = germfree, L = Lactobacilli, EC = E.coli, XGF = exgermfree, CV = conventional.

Paper V

Water consumption averaged 24,5 ml per animal per day. Corresponding to daily intakes of amoxicillin / clavulanic acid 100 / 25 mg / kg body weight.
Bacterial numbers were significantly lower in the amoxicillin / clavulanic acid treated group on both the operation day and the evaluation day (p=0.04) (Table 7).
The group treated with amoxicillin / clavulanic acid (group A) differed significantly from the placebo group (group B) regarding adhesion score (Mann-Whitney U p=0.019) as well as incidence (Fisher's exact test p=0.034) (Fig. 5).

Fig. 5. Adhesion score



A: amoxicillin / clavulanic acid
B: placebo

Table 7. Results of bacterial counts

Group	A	B
Operation day	10^{6-7}	10^{10-11}
Evaluation day	10^{6}	10^{10-12}

Discussion

As more research is being performed and the molecular events are beginning to be unraveled the complexity of adhesion formation increases (Holmdahl & Ivarsson 1999).

Complicating the current theory of the pathogenesis is the puzzling paradox that although hampered fibrinolysis is considered central in the formation of adhesions, they can be reduced by adding fibrin (Jahoda 1999, Holmdahl & Ivarsson 1999, Toosie et al 2000 and many more) Adding fibrinogen (Nisell & Larsson 1978) or fibrin does not increase adhesion. Furthermore, the application of thrombin does not increase adhesions (McGaw et al 1988, Wiseman et al 1992, Yarali et al 1998).

Studies have shown an adhesion-reducing effect both with anticoagulants, such as heparin, dicumarol, plasmin, streptokinase, tissue plasminogen activator (Buckman et al 1976a, 1976b, Thomson et al 1989, Menzies & Ellis 1989, Doody et al 1989, Vipond et al 1990, Dorr et al 1990, Fukasawa et al 1991, Orita et al 1991, Menzies & Ellis 1991, Dunn & Mohler 1993, Evans et al 1993, Lai et al 1998, Hellebrekers 2000a), as well as procoagulants, such as fibrin sealant and aprotinin (Young et al 1981, Chalkiadakis 1985, Chung & Nagy 1988, Koltai & Gerhard 1990, Larsson et al 1986, Lindenberg & Lauritsen 1984, Lindenberg et al 1985, de Virgilio et al 1990, Caballero & Tulandi 1992, Chmielewski et al 1992, Sheppard et al 1993, De Iaco et al 1994, Takeuchi et al 1996, Ozogul et al 1998, Jahoda et al 1999). Fibrin sealant, also claimed to promote fibroblast growth, consisting of a mixture of fibrinogen and aprotinin which mixes with thrombin through the passage of the syringe, forms a fibrin clot. Nevertheless, it has been found in many studies that fibrin sealant decreases adhesion formation (Lindenberg & Lauritsen 1984, Lindenberg et al 1985, Larsson et al 1986, Chung & Nagy 1988, Koltai & Gerhard 1990, de Virgilio et al 1990, Donnez & Nisolle 1991, Bothin & Hallberg 1992, Caballero &

Tulandi 1992, Chmielewski et al 1992, Sheppard et al 1993, Frykman et al 1993, De Iaco et al 1994, Boris et al 1996, Takeuchi et al 1996,1997, Ozeren et al 1998, Jahoda et al 1999, de Virgilio et al 1999, Moro et al 1999, Toosie et al 2000, Hellebrekers et al 2000b, Clark 2000, Meek et al 2001), which is rather counterintuitive in regard to the present theory of the pathogenesis. Intuitively, if adhesions could be prevented by only manipulating fibrin, it should be possible to design experiments where no adhesions at all form (Menzies 1992). However, no study, except for one (Villavicencio 1979) has accomplished this and not even defibrogenation by snake venoms resulted in zero adhesions (Ashby et al 1970, Buckman et al 1975, Chowdhury & Hubbell 1996), neither has plasmin (Gustavsson et al 1955, Knightly et al 1962). In addition, antithrombin did not abolish adhesions (unpublished data).

Studies have shown a reduction of peritoneal fibrinolytic activity and / or tPA-activity in conjunction with surgery, or other adhesion-inducing stimuli, and adhesion formation (Gervin et al 1973, Buckman et al 1976a+b, Hau et al 1979, Raftery 1981, Vipond et al 1990, Scott-Coombes et al 1995, Holmdahl et al 1996, Holmdahl 1997, Ivarsson et al 1998, Falk et al 2001), but there are quite many studies, on adhesion formation, or adhesion-inducing stimuli, that show normal or an increase in fibrinolytic and / or tPA-activity (Dorr et al 1992, Bakkum et al 1996, Edelstam et al 1998, Reijnen et al 2000, Hellebrekers 2000c, Reijnen et al 2002).

There could be situations in which the mechanism of adhesion formation is different. The notion that there are different types of adhesions has been put forward (Menzies 1992).

In (I) germfree rats were compared to their conventional counterparts regarding the response to an adhesion inducing stimulus. It was found that the GF animals had a

significantly weaker response regarding incidence as well as severity of adhesions compared to the CV rats.

The findings indicate that the bacterial bowel flora is of importance in postsurgical adhesion formation in CV rats and proves that it is not necessary for adhesion formation in GF rats. As the difference between the GF and the CV rats is the presence, in the latter, of an indigenous bacterial flora comprising more than 400 different bacterial species, it seems reasonable to assume that factors derived from the microbes of this flora are involved in the response of the CV rats. The observation of a weak adhesion response of the GF animals indicates that other mechanisms inherent in the animal itself are at hand, e.g. the possible ischemic part of the experimental lesion (Ellis 1962).

In (II), a study of a different species, DA-rats, it was found that the GF rats formed significantly less adhesions than their XGF counterparts. Thus, by establishing an indigenous bowel flora the ability to form adhesions returns. Again, the GF animals were not devoid of adhesions, indicating that the bowel flora is not the only factor involved and that more than one mechanism might be operating in this setting.

In (III) the effect of vitamin-A deficiency in GF and CV rats was studied regarding healing of colonic anastomoses and adhesion formation.

It has been reported that supplemental vitamin A increased adhesions in mice (Demetriou et al 1974). On the other hand, studies have reported an adhesion-decreasing effect by retinoic-acid administered postoperatively at the site of the adhesion-inducing lesion (Rodgers et al 1998).

We found, however, that vitamin A was important for the healing of colonic anastomoses irrespective of presence or abscence of the intestinal flora, and that the intestinal bacteria had a greater effect than the vitamin A-status on adhesion formation. This may indicate that the mechanisms of healing in colonic anastomoses and adhesion formation are different. A reflection that could be pondered upon is that any agent which retards wound healing will most probably stimulate adhesion formation.

Additionally, in (IV), germfree(GF) rats were compared to conventional (CV), exgermfree (XGF) and rats monocontaminated with E.coli (EC) and Lactobacilli (L) regarding the response to an adhesion inducing stimulus, i.e. colonic and ileal anastomoses. It was found that as the microflora approached the state of normal the more adhesions were seen, indicating different adhesiogenic ability of different bacteria. Different adhesiogenic ability has been observed by few authors (see Krook 1947, Yale & Balish 1992). Restoration of the microbial environment restored the adhesion forming ability.

In (V) antibiotic treatment resulted in lower bacterial counts and less adhesions than in the placebo group. This is interpreted as when the flora pressure is suppressed by antibiotics less adhesions are formed.

The intention for giving amoxicillin / clavulanic acid was to have an absorbable compound being partly protected for bacterial enzymatic breakdown within the intestinal lumen (clavulanic acid) and with a possible effect upon the gram-negative as well as gram-positive part of the flora. Additionally, the systemic availability could be more advantageous than non-absorbable compounds because the drug is present in the tissues constituting the

interface between lumen and host. In contrast to non-absorbable drugs which may in fact contribute to translocation due to the resulting unbalanced flora.

The present study shows that suppression of the intestinal flora with antibiotics decreases adhesions, consistent with findings by other investigators (Brolin et al 1984, Videla et al 1994, Oncel et al 2001).

Antibiotics are widely used in clinical surgery and may therefore influence adhesion outcome. Ongoing pilot studies have indicated that resistance to antibiotics leading to dominance of gram-negative organisms may drive the process towards more adhesions.

The influence of the bacteria could have been overlooked in the past and might have been a contributing factor, at least in part, for previous contradicting results between different studies. There might even be microflora-associated adhesions and host-associated adhesions.

How the bowel flora effect is brought about is unknown but could be due to translocation of bacteria or leakage of bacteria-associated products, stimulating adhesion formation. No overt signs of contamination or infection of the operating field was seen in the animals. However, it is well established that microbes translocate from the intestinal tract under physiological conditions (Deitch 1990). Several conditions such as hemorrhagic shock, burns, mechanical trauma etc may cause increased translocation (Berg 1992). Most likely, the adhesion inducing stimuli used in this study may initiate a similar increase in bacterial translocation, which in turn may influence the formation of adhesions. In the anastomosis model translocation of bacteria or simple leakage or migration along sutures or through small deficiencies may stimulate adhesion formation.

Other possibilities include bacterial influence on the immune system or by modifying the tissue response to the surgical trauma. It might also be via influence on healing mechanisms (Okada 1994, Liu et al 1996) or possibly on the fibrinolytic system.

Interestingly, in the germfree animals there was not a complete lack of adhesion formation, indicating that factor(s) derived from the animal itself may cause adhesions, e.g. the possible ischaemic part of the experimental lesion (Ellis 1962).
The combination of a microbial influence and ischemia may be the cause of adhesions frequently encountered around bowel anastomoses in clinical surgery.

Moreover, these results are interesting because they may help to explain why tissue plasminogen activator works on side-wall adhesions but not around anastomoses and ischemic bowel (Menzies & Ellis 1991). There is also a notion of two different types of adhesions, one in which fibrinolysis is crucial and that is readily prevented with for example recombinant plasminogen activator (rt-PA) and one which occurs around colonic anastomoses and to ischemic small intestine where rt-PA has no effect (Menzies 1992).

Attempts to explain the fibrin paradox is lacking. However, there seems to be a link between bacteria and fibrinolysis. Studies have shown that some tested bacteria species can bind plasminogen and increase its activation to plasmin by a factor of more than a hundred (Eberhard et al 1995). These bacteria use the proteolytic effect of plasmin to penetrate tissue barriers (Virkola et al 1996, Eberhard et al 1999).
It could be surmised that bacteria use the very systems that protect against adhesions for

their virulence and that the decrease in fibrinolysis known to occur in conjunction with adhesion provoking events are deviced to incur adhesions to protect the host against bacterial invasion.

Although most postsurgical adhesions are harmless as they form so commonly after surgical procedures, they are certainly known to incur a great deal of morbidity in the form of infertility in women, mechanical obstruction, technical difficulties in subsequent operations and other complications. However, adhesions probably have a survival value in walling off potential leakage of bacteria as for instance in perforations of natural causes as well as around leaking surgical anastomoses (Echtenacher et al 2001).

Adding fibrin in this situation may work by containing / walling off the bacteria. It could be speculated that fibrin, indeed, has a dual role in adhesion formation.

Some bacteria species are supposedly stronger promotors of the adhesion forming process, as indicated in (IV). Antibiotics may reduce adhesions but may possibly also lead to selection and overgrowth of potentially adhesiogenic species due to resistance problems. Whether probiotic manipulation with for instance bulk or lactobacilli could be beneficial is as yet unknown but should be explored.

The emerging interplay between bacteria and host may help in the quest for understanding the adhesion forming mechanism. Further investigations are warranted on the influence of the microflora and its interactions with host tissues and adhesion formation. The germfree state could be used as a 'clean' model in adhesion research, void of the bacterial influence.

Scoring of adhesions(II)

¨How much of a reduction in post-operative adhesions is necessary before it is clinically relevant? A single adhesion in the wrong anatomic location may be catastrophic. How do we measure this?¨ (Johns A 2001).

Measuring adhesions is difficult. The best end point would be to record whether adhesions are present or not (on / off - method), however this can seldom be used because no one of all the tested measures to date totally abolishes adhesion appearance. Many publications demonstrate a reduction in grade but not the number of adhesions, whereas others show a reduction in frequency. Most of the different scoring systems comprise categorizing the adhesions involving an element of subjectively distinguishing between adhesions according to severity. Others have measured for instance the width of adhesions or the percentage of a surface area covered by adhesions, but it is not certain that these methods truly identifies a stronger biological response.

Consequently an attempt to develop an objective, less observer-dependent, scale was made (II, revised V). This scale does not involve the element of subjectively grading adhesions by severity, it merely counts events, in effect the number of on / offs. The idea is that a large sum of events is more likely to represent a stronger response than a small sum of events. The new scale, meticulously constructed during a great many operations,

is easy to use and should be simple to employ by others wishing to do so. Eventually, it could lead to improved possibilities to compare the results of different studies.

Conclusions

- The bacterial flora of the gastrointestinal canal influences adhesion formation but is not essential for adhesions to develop.

- Restoration of an ordinary flora restores adhesion forming propensity.

- Vitamin A is important for healing of colonic anastomoses but does not affect adhesion formation whereas the intestinal flora status does, indicating that the mechanisms might be different.

- The more normal the flora gets the more normal the adhesive response, indicating that different species of bacteria have different adhesiogenic ability.

- Antibiotics lowering bacterial numbers of the gastrointestinal flora reduce adhesions, but resistance problems may theoretically induce growth of potent adhesiogens.

- The new, less observer-dependent, event-counting scoring scale, was as good as scales compared at detecting differences , but has advantage in the form of being objective.

Summary

In this thesis four of the papers, evaluated adhesion formation in germfree rats and one paper dealt with the effect of antibiotics. The former papers are the only ones of its kind reported in the PubMed and the latter has one compatriot (Oncel et al 2001) and two papers with observations of the effect of antibiotics (Brolin et al 1984, Videla et al 1994).

Taken together, the studies have, unequivocally, shown that the intestinal microflora plays a role in adhesion formation and that antibiotic manipulation can reduce adhesions, but supposedly not beyond the level of germfree rats, and that antibiotics may, theoretically, promote growth of adhesiogenic germs and thereby aggravate adhesions.

In addition, germfree rats will be an invaluable resource for further studies on adhesion mechanisms as they are void of the bacterial influence and can be used as a 'clean' model.

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Occam's razor

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