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EVALUATION OF ANTIBODY CLASSES PRODUCED IN RESPONSE TO TREATMENT WITH CONTIGEN® IMPLANT

A Thesis

Presented to

the Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements of the Degree

Master of Science

by

Marcee M. McClelland

December, 1996

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ABSTRACT

EVALUATION OF ANTIBODY CLASSES PRODUCED IN RESPONSE TO TREATMENT WITH CONTIGEN® IMPLANT

By Marcee M. McClelland

This thesis address the characterization of antibodies produced in response to treatment with Contigen® Bard® Collagen Implant (CI) for urinary incontinence. CI is a highly purified bovine dermal type I collagen (BDC). In a prospective clinical study, 382 patients were treated with CI in the urinary sphincter and blood was collected at various timepoints following injection. Approximately 28% of the patients treated with CI demonstrated specific antibodies against BDC. Serum samples from 27 of the patients from this cohort who had samples available at multiple timepoints were evaluated. The class specificity of circulating antibodies against bovine collagen was characterized using an indirect enzyme-linked immunosorbent assay.

In all patients demonstrating an antibody response to bovine collagen, the predominant immunoglobulin class was IgG, found in 100% of serum samples. IgA was produced in approximately 40% of these patients, and IgM was detected in approximately 0.6%. No specific IgE was detected against bovine collagen in any serum sample.

Acknowledgements:

I would like to thank Dr. Ruthann Kibler for providing the support and encouragement necessary to complete this thesis manuscript and for making my San Jose State University experience a good one. Thank you also to Dr. John Boothby for his support and review of this manuscript. Finally, I would like to thank Dr. Frank DeLustro, who has given me the confidence, encouragement and support I needed to make this project a reality.

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Historical Significance of Thesis Research

Urinary incontinence affects millions of people worldwide. This condition, which is defined by involuntary loss of urine, has social, emotional, economic, and physical side effects. A novel biomaterial, Contigen® Bard® Collagen Implant (CI), was evaluated for effectiveness as an incontience treatment.

Contigen® Bard® Collagen Implant is a biocompatible, gluteraldehyde cross-linked bovine dermal collagen composed of approximately 95% type I collagen with 5% or less type III collagen. Contigen is used as a locally injected bulking agent which causes subsequent coaptation of the urethral lumen. The concept of using bovine dermal collagen to augment deficient urinary sphincters in order to increase outflow resistance was a result of this material's ability to augment soft tissue contour irregularities.

Bovine collagen has been safely used for many years in the treatment of facial scars and defects (1-5). Early immunologic studies of type I bovine collagen found the telopeptide ends of the molecule to be the most immunogenic (6,7). During the processing of CI these telopeptide ends are removed through proteolytic digestion. The biochemical characterization of crosslinked and non-crosslinked

collagens has been extensively reviewed, especially for soft tissue applications (8-10). Crosslinking effectively stabilizes collagen materials resulting in decreased biologic degradation and increased persistence compared to non-crosslinked materials in soft tissue augmentation applications (9,11). Gluteraldehyde crosslinking has also been shown to reduce further the immunogenicity of collagen biomaterials (12) and to enhance the biocompatibility by encouraging fibroblast infiltration and neovascularization (8,9).

A multicenter open evaluation study was undertaken to evaluate CI in the treatment of urinary stress incontinence. Patients were treated with up to 30 cc of CI via a transurethral cystoscope (13). The cause of the patients' incontinence was recorded upon enrollment in the study. Data was collected on the amount of material that was used, the incontinence grade prior to and after treatment with CI, and the presence of circulating antibodies against bovine dermal collagen prior to treatment, as well as antibody production after subsequent treatment. The objective of the study was to examine the safety and effectiveness of the use of CI in the treatment of urinary incontinence. All patients enrolled in the study had been incontinent for at least three months prior to treatment.

The etiologies of the incontinence were due to irreversible pathologies that affected the function of the bladder sphincteric mechanism (14). The best results were seen in patients with incontinence following radical prostatectomy, myelodysplasia or spinal cord injury, and intrinsic sphincter deficiency (ISD). All of these conditions are permanent, and therefore any improvement experienced by the patients in their incontinent conditions came as a direct result of treatment with CI.

In the multicenter clinical study to evaluate CI, cited above, development of immune responses to bovine collagen were monitored (15). All patients were screened for hypersensitivity to bovine collagen using a skin challenge in the volar surface of the forearm. Patients demonstrating a positive skin challenge response were excluded from study participation. Development of bovine collagen antibodies was monitored throughout the study. Blood samples were collected from patients before treatment, at the time of treatment and 1,3,6 and 12 months post-treatment. Blood samples were sent to SmithKline Beecham Clinical Laboratories (Venice, CA) for serum isolation and storage until evaluation. Samples were

evaluated for bovine collagen antibodies using indirect enzyme-linked immunosorbent assay (ELISA)(16).

Of the 347 patients evaluated for antibodies, 17 patients demonstrated bovine dermal collagen specific antibodies before treatment. In these patients antibodies were present throughout the course of treatment at all timepoints tested. For patients not demonstrating bovine specific antibodies before treatment, but who eventually demonstrated antibodies, the majority developed a significant titer 1 to 3 months following initial treatment. Serologic analysis of all patients showed no correlation between the onset of an adverse event and the presence of circulating antibodies (15). According to the Food and Drug Administration (FDA) guidelines, all adverse events were recorded regardless of whether they were suspected to be associated with CI treatment or were believed to be unrelated to treatment. There was also no correlation found between antibody production against bovine dermal collagen and treatment effectiveness.

This thesis evaluates the immunoglobulin (Ig) class specificity (IgG, IgA, IgM, or IgE) of the antibodies produced in reponse to treatment with CI. Ig class characterization can lead to a better

understanding of what is occurring clinically in response to a specific antigen. For example, IgM is a very potent initiator of the classical pathway of the complement system (17). IgM is approximately 600 times more efficient in complement binding than IgG, requiring only a single IgM to initiate the cascade, and cause subsequent cell toxicity and lysis (18). Certain cells, including red blood cells, white blood cells, platelets, and vascular endothelium, are especially susceptible to the effects of complement (18). IgE is of particular clinical significance due to its involvement in allergic reactions. The production of IgE, and its subsequent binding to mast cells and basophils, is known to be the primary mediator of anaphylaxis (17). IgA is associated with mucosal immunity and may occur through gut sensitization (17). The objective of this thesis research was to discover which Ig classes are produced in response to CI treatment, at what timepoints over the course of treatment, and in what quantities (19).

Evaluation of Antibody Class in Response to Bovine Collagen Treatment in Patients with Urinary Incontinence

by

Marcee McClelland

San Jose State University

Abstract

Purpose: Contigen® Bard® Collagen Implant (CI), made of highly purified bovine dermal type I collagen (BDC), is used as a bulking agent for the treatment of urinary stress incontinence. The humoral immune response to placement of this material in the urinary sphincter was evaluated.

Materials and Methods: In a prospective clinical study, 382 patients were treated with CI in the urinary sphincter and blood was collected at various timepoints following injection. Approximately 28% of the patients treated with CI demonstrated specific antibodies against BDC. Serum samples from 27 of the patients from this cohort who had samples available at multiple timepoints were evaluated. The class specificity of circulating antibodies against bovine collagen was characterized using an indirect enzyme-linked immunosorbent assay.

Results: In all patients demonstrating an antibody response to bovine collagen, the predominant immunoglobulin class was IgG, found in 100% of serum samples. IgA was produced in approximately 40% of these patients, and IgM was detected in approximately 0.6%. No specific IgE was detected against bovine collagen in any serum sample. The highest concentrations of IgG and IgA antibody classes were observed at 4 to 5 months after the initial treatment with CI. In the multi-center clinical trial, adverse events were

reported in approximately 40% of all patients treated with CI (19). There was no correlation found between the production of a specific immunoglobulin class and the onset of any clinical adverse events.

Conclusions: In patients treated with CI for urinary stress incontinence, who developed antibodies to bovine dermal collagen, the predominant immunoglobulin class was IgG. IgA was seen in less than half of the sera samples, and IgE to bovine dermal collagen was not observed. In addition, no change in the humoral response to CI over time was noted in patients demonstrating pre-sensitization to bovine dermal collagen at the time of initial treatment. Clinical adverse events reported for patients demonstrating pretreatment antibodies against bovine dermal collagen did not differ in type of events or number of events when compared to patients with no pre-sensitization.

Key Words: Urinary Incontinence, Collagen, IgG, IgM, IgA, IgE

Introduction

Injectable bovine collagen has been used safely and effectively as a biomaterial for dermal augmentation for 20 years. (3,4,20) The biocompatibility of this material in humans has been demonstrated in clinical trials and clinical use (20-22).

Contigen® Bard® Collagen Implant (CI) is an injectable purified type I bovine dermal collagen, cross-linked with glutaraldehyde (15). A multi-center, open-label evaluation of CI was undertaken to assess the efficacy of this material in the treatment of urinary incontinence. Serum samples were acquired from the 382 patients treated in this study. The study protocol called for blood to be drawn before the skin test, on the day of treatment, and at 1,3,6,12, and 24 months after treatment. The timing for patient follow-up visits was not strictly adhered to and, therefore, serum was drawn at different timepoints for different patients. Positive clinical results for the use of glutaraldehyde crosslinked collagen for the treatment of deficient urinary sphincters have been reported from this study (23-25). The most promising clinical results have been seen in female patients with intrinsic sphincter deficiency (ISD). Elevated antibody titers against bovine type I collagen have been reported in patients treated with CI (15). The objective of this study was to further characterize the humoral response to CI.

Antibodies produced in response to treatment with CI were evaluated for class specificity using an enzyme-linked immunosorbent assay (ELISA).

Approximately 5% of all patients treated with CI had pre-existing antibodies to bovine dermal collagen (BDC), and another 23% produced antibodies following treatment. Serum samples from 27 of the 382 patients enrolled in the multi-center open label evaluation of CI were evaluated using the ELISA method. All 27 patients produced antibodies against BDC at least one time point after injection with BDC. The immunoglobulin (Ig) class distribution was evaluated for 181 serum samples from this 27 patient cohort. Serum samples were obtained at multiple time points before, during, and after treatment with CI; these sera were evaluated for Ig class and concentration.

Materials and Methods

1) Patient Selection

All patients used in this evaluation were treated with CI in an multi-center open label evaluation (13). The criteria for patient selection in this study have been previously described (13). In short, all 382 patients treated with CI had been incontinent for at least three months prior to injection. The incontinence was due to irreversible pathologies that affected the function of the bladder sphincteric mechanism. Patients were excluded from the study if they had untreated urinary tract infection, unmanaged detrusor instability, or known hypersensitivity to BDC. An average total volume of 28.9 cc for women and 74.9 cc for men (up to 30cc per treatment) of CI was injected into the urethral submucosa in these patients with deficient urinary sphincters.

All serum samples were evaluated for antibodies against BDC. One hundred and eighty-one samples from 27 of the positive patients from this clinical study were evaluated here for Ig class and concentration.

2) Immunoassays

The assay for the detection of Ig classes utilized two immunologic techniques, the sandwich ELISA and the indirect ELISA. The sandwich ELISA was used to establish a standard calibration curve with human Igs of a known class and concentration (26). The indirect ELISA was used to evaluate the Ig class produced in response to treatment with CI. It was necessary to utilize these two immunoassay techniques because characterized class-specific anti-bovine collagen antibodies were not available to use as controls for the indirect ELISA for IgM and IgE.

The presence of antibodies against bovine dermal collagen was determined as described by Ellingsworth et al. (16). In short, an indirect ELISA was performed by coating BDC, dissolved in 1% acetic acid, onto a polystyrene 96 well microtiter plate (Dynatec, Chantilly, VA). The BDC was coated at a concentration of 2.5 ug/well. Following an overnight incubation at 4°C, the plates were washed four times with phosphate buffered saline (PBS) containing 0.05% Tween-20, and blocked with 0.01 M PBS (pH 7.2) containing 0.05% Tween-20 and 1% (w/v) bovine serum albumin (BSA) for 1 hour. Each patient's serum sample was two-fold serially diluted (1/20-1/2560) in 0.01 M PBS (pH 7.2) containing 0.05% Tween-20 and 1% (w/v) BSA, and

added to duplicate wells. After 2 hours, the plates were washed four times with PBS containing 0.05% Tween-20, and 100 ul of peroxidase-conjugated rabbit anti-human IgG, IgM, IgA, or IgE antibodies (Dako Corp., Carpinteria, CA) was added to each well for 1 hour. The plates were again washed 4 times, and 100 ul of 2,2'-azino-di-(3-ethly-benzthiazoline) sulfonic acid (ABTS; Sigma Chemical Co., St. Louis, MO) was added to each well. The reaction was allowed to develop for 30 minutes and the optical density was read at 414 nm against a background wavelength of 520 nm (EMAX, Menlo Park, CA).

The sandwich ELISA utilized the same method as the indirect ELISA except for the antigen coating and the primary antibody incubation. Rabbit anti-human IgG, IgM, IgA, or IgE antibodies (Dako Corp., Carpinteria, CA) were coated to specific wells used for the calibration curve at a concentration of approximately 1.0 ug/well. The plates were incubated overnight at 4°C, washed 4 times as previously described, and incubated for 1 hour with 0.01 M PBS (pH 7.2) containing 0.05% Tween-20 and 1% (w/v) BSA. Human IgG, IgA, IgE (Calbiochem Corp., La Jolla, CA) or IgM (Jackson Immunoresearch Laboratories, West Grove, PA) was diluted from 500 ug/L to 20 ug/L in 0.01 M PBS (pH 7.2) containing 0.05% Tween-20 and 1% (w/v) BSA and added to duplicate wells for 2 hours. The plates were then washed 4 times and the rest of the ELISA was performed as the indirect ELISA, beginning at the addition of peroxidase conjugated rabbit anti-human Ig.

3) Sample/Statistical Analysis

Antibody class titer was defined as the lowest dilution at which the absorbance value measured was greater than the established cut-off for each antibody class. The cut-off was established using human serum from 30 patients known to be negative for antibodies against BDC, plus 2 standard deviations (SD). The concentrations of the Ig classes in patients' serum were determined using a four parameter curve fit of the standard human Ig of known concentration and class specificity. A patient sample was considered negative for a given antibody class if the concentration measured was below the average concentration present in pooled normal human sera + 2 SD. A t-test for independent samples (with equal/unequal variances) was used to test for the significance between the mean of two groups.

Results

The detection limit for IgG, IgA, and IgE was 20 ug/L; for IgM, it was 40 ug/L (fig. 1). No cross-reactivity among antibody classes was observed. The sensitivity of the assay has been shown to be adequate for measuring normal to elevated levels of Ig in all classes (27).

Of the 181 samples taken at different timepoints from 27 patients, 162 demonstrated antibodies against BDC (titer ≥40). All 162 sera contained antibodies of the IgG class specific for BDC (fig. 2). IgA against BDC was demonstrated in 40.4% of all positive sera samples, and IgM specific for BDC

was seen in 0.6% of the same samples (fig.2). There was no IgE produced at any time point in any of the patient sera evaluated (fig. 2). Anti-BDC antibodies of the IgG class were present in sera of all 27 patients evaluated (100%), IgA to BDC was seen in sera of 12 patients (44%), and IgM to BDC was observed in serum of 1 patient (3%).

The presence of Ig classes specific for BDC was evaluated in multiple samples from each patient to establish the trend of antibody production for each Ig class over time. The IgG concentration peaked at 4.8 months after the initial treatment, and 2.5 months after the last treatment with CI. The IgA peak occurred 4.0 months after the initial treatment, and 2.7 months after the last treatment with CI (fig. 3). Of the 27 patients with antibodies to BDC, 6 demonstrated pre-existing (pre-treatment) IgG specific for bovine collagen. Of these 6 patients with pre-existing IgG, only 1 patient demonstrated an antibody response significantly different than the patient cohort without pre-existing IgG (fig. 4). Only 1 patient demonstrated pre-existing IgA specific for bovine collagen. There was no significant difference in antibody response in this patient when compared to the rest of the patient cohort (fig. 5). The mean peak antibody concentrations seen among sera from all patients is shown in table 1. There was no statistically significant difference in the mean antibody concentration seen in patients with pre-existing IgG against BDC compared to those without pre-existing IgG against BDC (p=0. 35). The mean IgG concentration in the pre-existing antibody group decreased from 25.2 ug/L

(s.d = 37.1) to 11.45 ug/L (s.d. = 8.07) when the statistical outlier, patient #S023 (see fig. 4) was removed from the pre-existing antibody group.

Discussion

The immune response to injectable bovine collagen has been well described over 15 years of clinical experience (1-5,12,14,15). Antibodies are produced in response to BDC in approximately 10.3% of all patients treated with this material (3). At least 2% of the population have pre-existing hypersensitivity directed against BDC (2). The antibody response to BDC varies with the treatment site and indication (22). The reason for this is unknown, but may be a result of the difference in average age for patients treated for different indications. The mean age of patients treated for urinary incontinence is much higher than the mean age for patients treated in dermal or orthopedic applications. The differences in the underlying etiology which led to treatment with BDC may also be a factor in subsequent antibody production. The incidence of clinical adverse events following treatment with BDC for soft tissue augmentation is approximately 1.2-1.3% of all patients treated (4,15). Antibody production occurs in approximately 90-100% of all patients exhibiting a hypersensitivity reaction to this material (2,4,22,30). Following intradermal collagen injection for the treatment of rhytids, clinical manifestations of a hypersensitivity reaction typically include erythema, induration, and/or pruritus at the implant site. These reactions usually last

between 4 to 6 months and resolve without treatment as the implant is resorbed (2,4). Antibody production does not necessarily lead to the onset of an adverse event. In fact, patients who do not have an adverse clinical event may still demonstrate circulating antibodies against BDC (15,22).

The objective of this study was to characterize in detail the humoral immune response to BDC following submucosal injection into the human urinary sphincter. Histology was evaluated in 7 patients who received subureteral injections of small volumes (0.95 cc average) of BDC. Biopsies were taken 3-19 months after treatment with BDC at the time of reimplantation. The local tissue reaction was reported to contain a minimal inflammatory response (28). Histologic evaluation of one patient treated with BDC in the urinary sphincter was also described as exhibiting only mild inflammation (31). Antibodies were produced in response to CI in approximately 28 % of all patients treated in the present clinical study, including those with pre-exsisting antibodies.

The class distribution of these antibodies was evaluated in sera of the patient cohort with anti-collagen antibodies. The antibody class produced most often and in the greatest concentration was IgG, which was found in 100% of the patients evaluated. IgA specific for BDC was produced in 44.4% of the patients (12/27). These findings are consistent with earlier studies of Frank et al. (30) and DeLustro et al. (29), who found that patients first demonstrating antibodies against bovine collagen demonstrated mainly IgG

specific for this material, followed by IgA produced in one-third to one-half of the patients with hypersensitivity to BDC. There was no statistically significant difference ($p>0.05$) in volumes of CI used to treat those patients who developed IgA specific to bovine collagen compared to those patients whoh did not. In the current study, we observed the peak IgG response at 2.5 months after the last treatment with CI and the peak IgA response at 2.7 months after the last exposure. A large standard deviation was seen for the average time point for both peaks. This was a result of the study design, which allowed repeated treatments with CI throughout the study for better clinical results.

One patient demonstrated low levels of IgM specific for BDC at 6 months post-treatment with CI, but IgM was not seen in this patient at any other time point. This patient received a total of 30cc of CI, which is not statistically different from the average total volume given to patients who did not develop IgM against BDC. Two clinical adverse reactions were reported for this patient: hematuria and urinary tract infection. Both of these clinical manifestations were reported commonly throughout the study, representing 2% and 20% respectively of all patients reporting events (32), and no correlation was found between antibody production and the onset of these events.

All patient samples evaluated were negative for IgE specific for BDC, as previously reported for patients treated with BDC for correction of facial

rhytids (29). The production of IgE, and its subsequent binding to mast cells and basophils, is associated with immediate hypersensitivity. An IgE response usually occurs at mucosal surfaces or local lymph nodes (17). Because CI is injected into the submucosa of the urethra, and because of the seriousness of possible clinical manifestations, the presence of IgE in response to bovine collagen is of great interest. Our data indicate that an IgE-response is not associated with the use of CI in the submucosal space of the urethra.

Pre-sensitization to bovine collagen is believed to occur by dietary exposure to this antigen (15,30). The effect of pre-existing IgA levels on the humoral response to bovine collagen is of interest because of the continual exposure of the gastrointestinal tract to bovine collagen through the consumption of beef products in the normal diet. Indeed, in the one patient with pre-existing IgA specific for this material, no significant difference was found in the IgA response to bovine collagen over time when compared with the entire patient cohort. The only clinical adverse event reported in this patient was intermittent urinary retention. This event has been reported frequently among patients treated with CI (32). In the patient with pre-existing IgA, the intermittent urinary retention resolved without medical intervention. One of six patients with pre-existing IgG specific for BDC exhibited a significantly higher antibody response against BDC. This patient reported urinary tract infections (UTI) four times over the course of the study. UTI was the most common adverse event reported in this study and was seen

in equal proportions among patients with and without antibodies against BDC. No other events were reported for this patient.

The serum samples evaluated here for class specificity were previously evaluated by indirect ELISA for cross reactivity with human type I and type III collagen. No cross reactivity was observed. Similar findings were reported in other clinical investigations (2,16,33). The presence of IgG, IgA, and in the case of one patient, IgM specific for BDC, showed no correlation with the onset of any unexpected adverse clinical manifestations. This characterization of antibodies produced in response to BDC indicate that CI can be safely and effectively used in the treatment of urinary incontinence, and is in agreement with the conclusions of others (13,15,23-25,28,31) .

Table 1. Mean peak antibody class concentration for positive sera in patients with and without pre-existing antibodies to BDC.

Sera Source	Measurements	Average IgG Concentration in mg/L	Average IgA Concentration in mg/L	Average IgM Concentration in mg/L	Average IgE Concentration in mg/L
Patients without pre-existing antibodies	Mean =	10.7†	3.2	1.01	Not Detectable
	n =	17	9	1	
	s.d.=	11.77	2.67		
	range* =	2.0-37.4	1.6-9.66		
Patients with pre-existing antibodies	Mean =	25.2†	2.3	Not Detectable	Not Detectable
	n =	7	1		
	s.d.=	37.1			
	range* =	2.6-107.7			

* the antibody concentration range is shown for each class and represents the high and low concentration peaks in mg/L.
† there is no statistically significant difference in the IgG concentration seen in patients with and without pre-existing antibodies to bovine collagen.

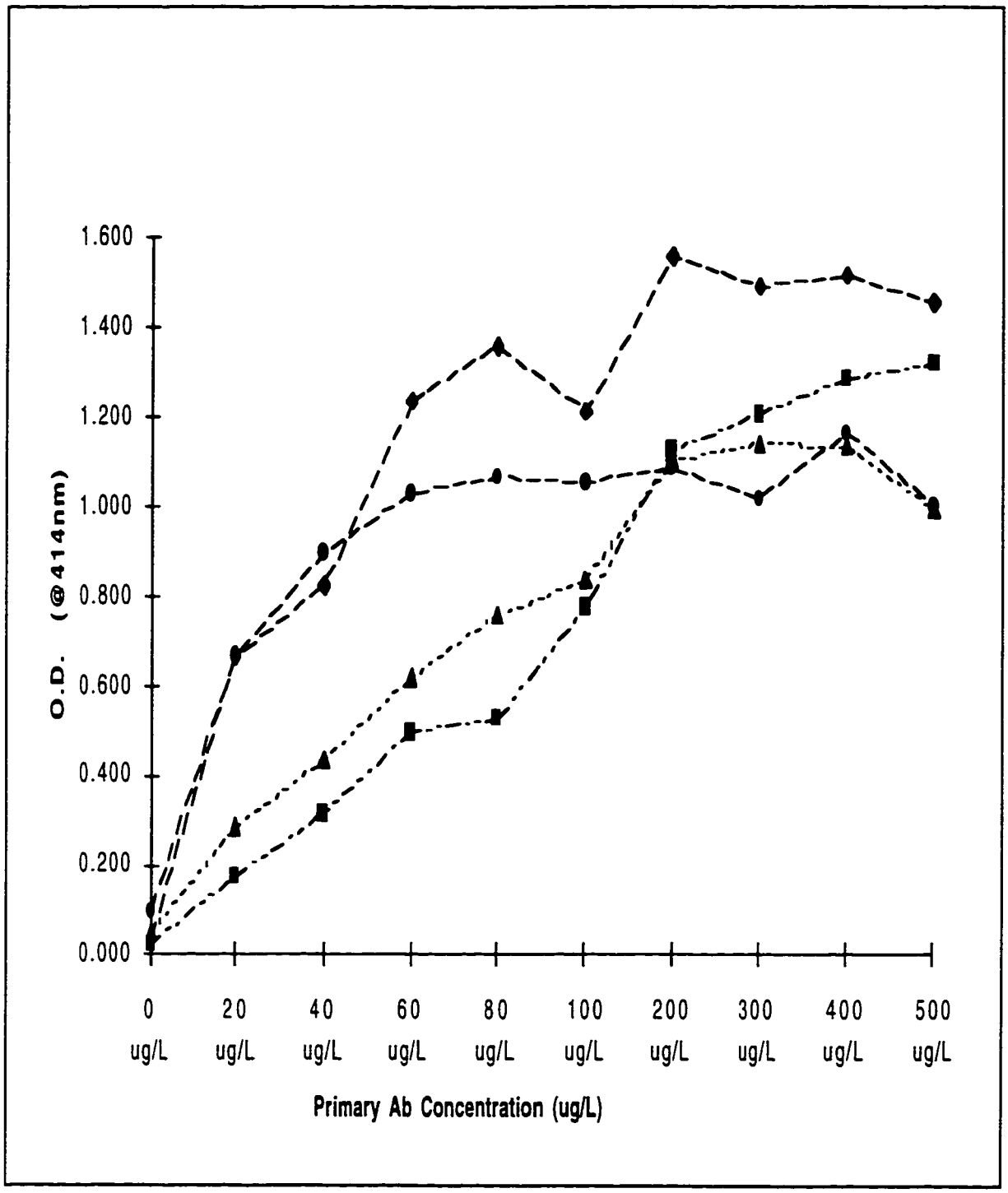

Figure 1. *Calibration curve of known antibody class and concentration.* The concentrations of IgA(◆), IgG(●), IgE(▲), and IgM(■) are in ug/L.

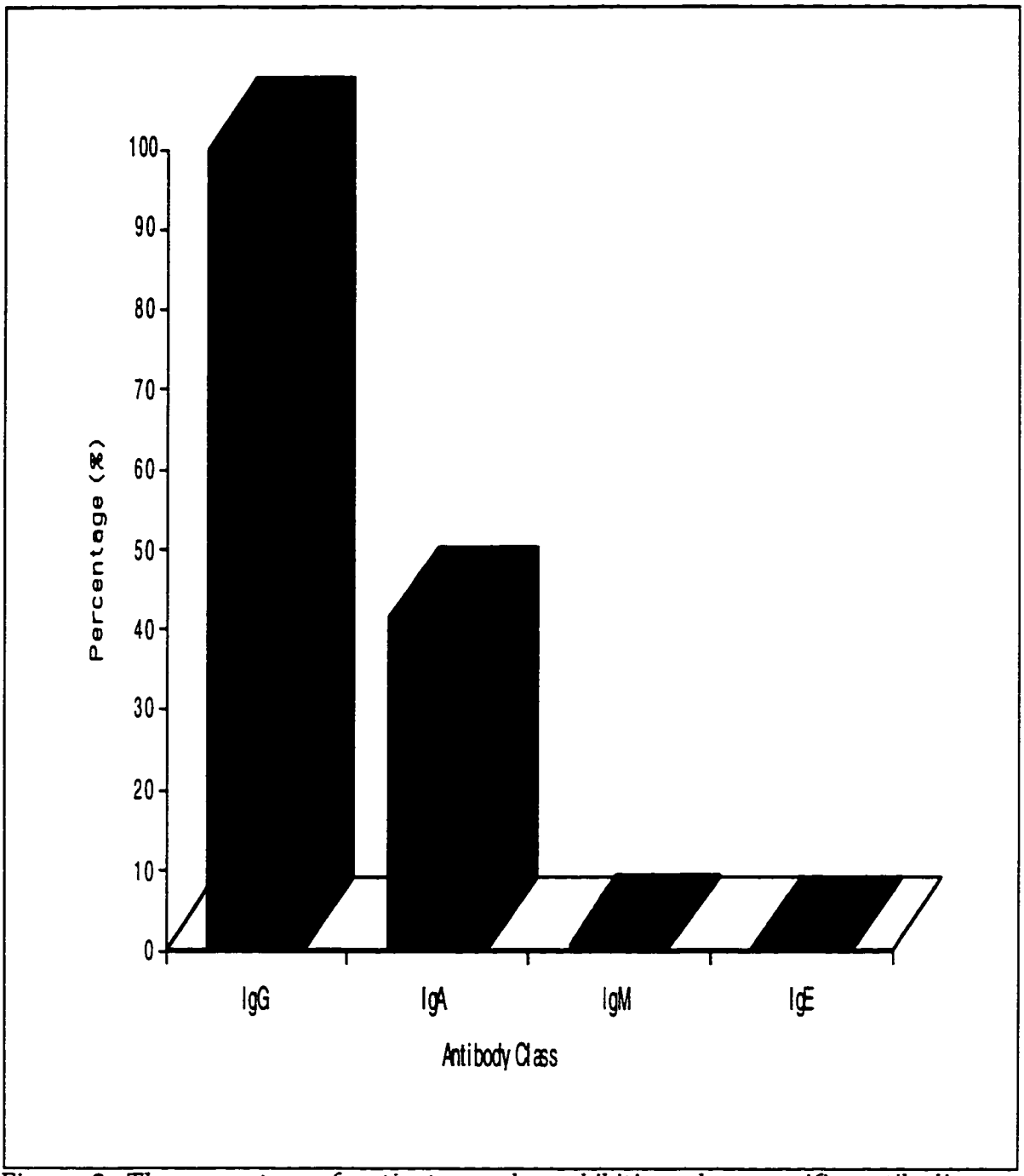

Figure 2. *The percentage of patient samples exhibiting class specific antibodies against bovine dermal collagen.* A positive titer is defined as a titer ≥ 40. The n-value of 162 represents all patient samples demonstrating antibodies against BDC in any antibody class.

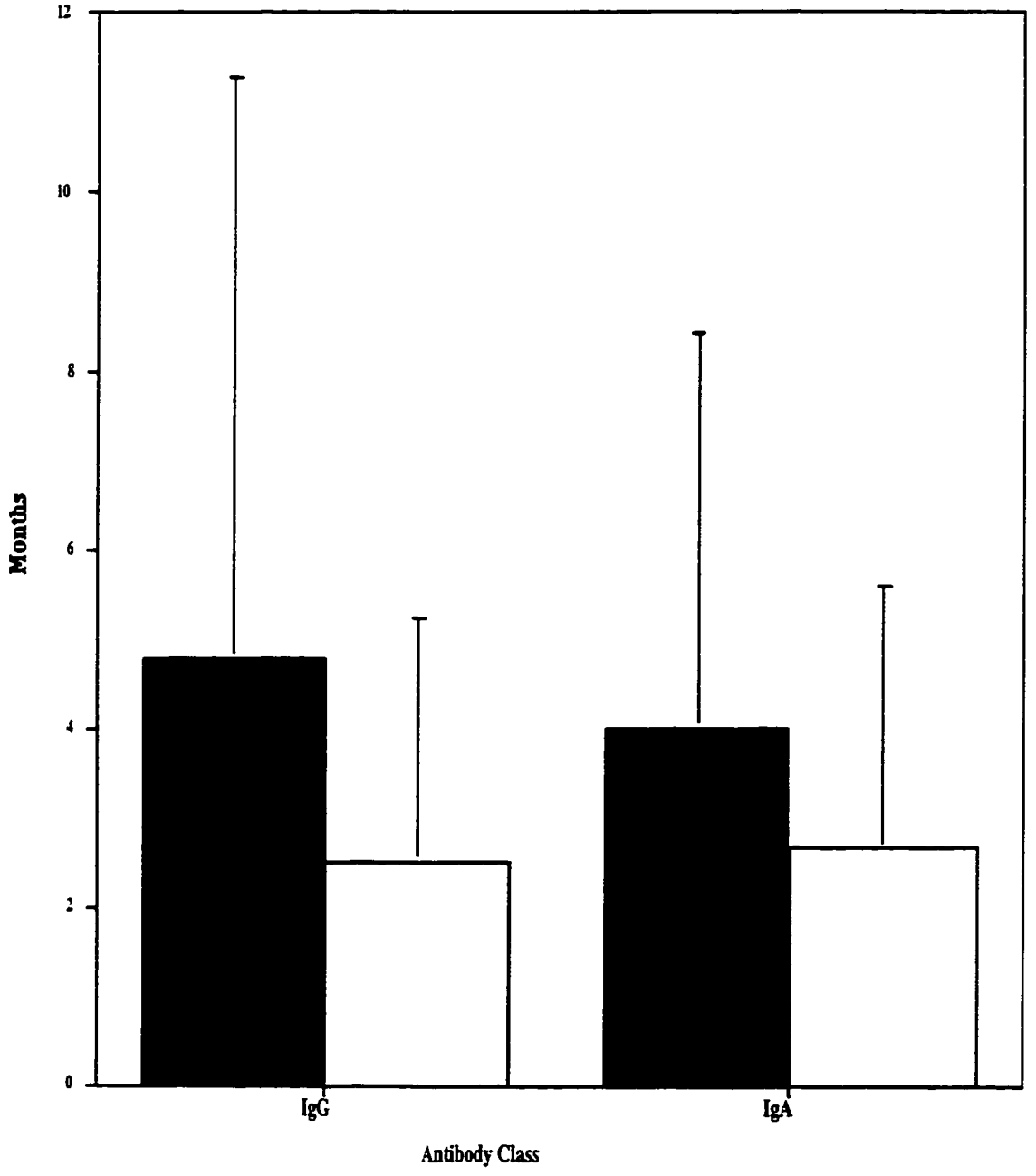

Figure 3. *The average time point at which an antibody peak occurs in patients treated with Contigen® Implant.* The antibody peak is defined as the highest concentration seen in each patient for each antibody class. The number of months since the initial treatment with CI (■) and the number of months since the last treatment with CI ( ) are both represented above. Standard deviations are represented by error bars.

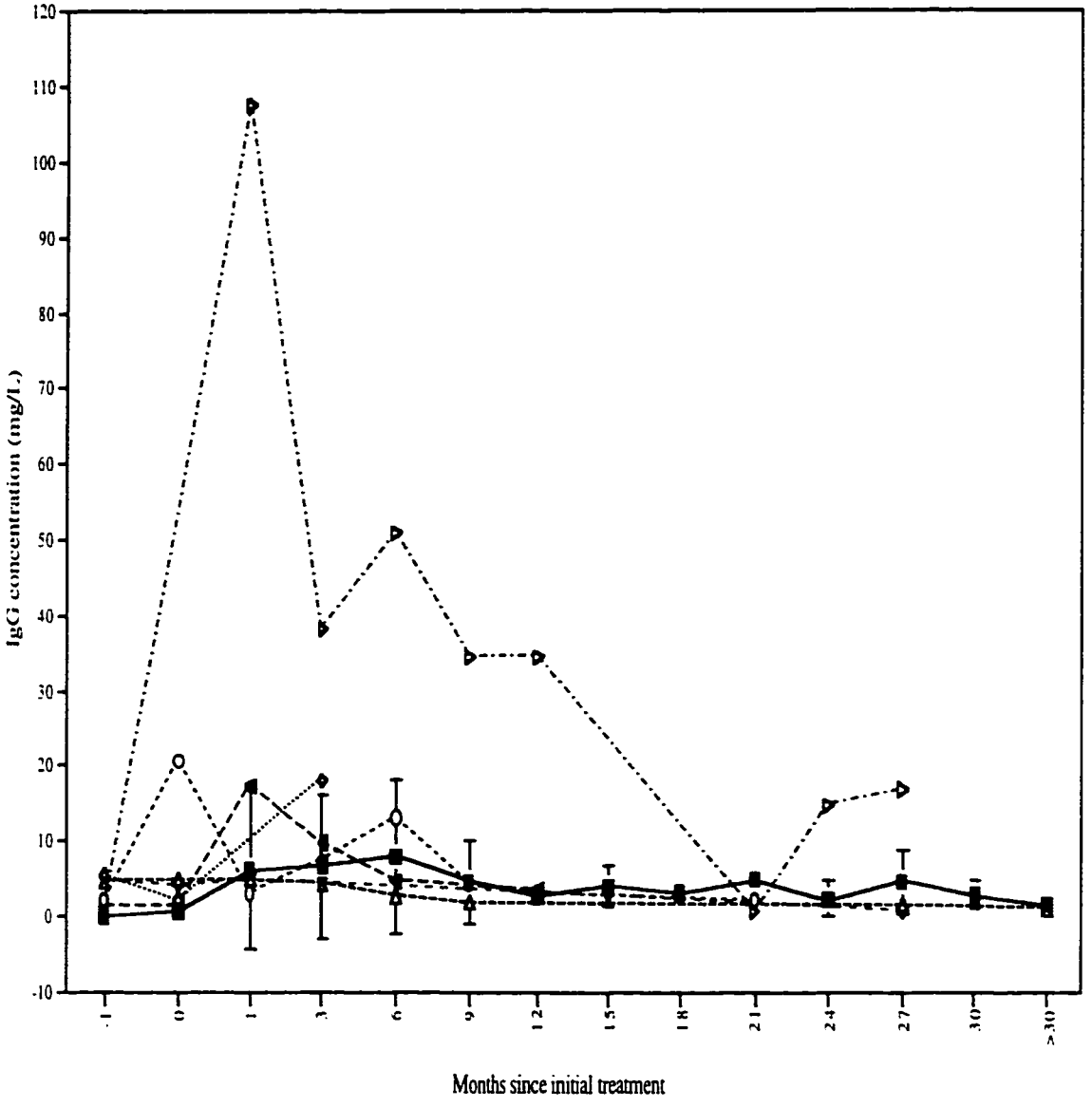

Fig 4. *The average Concentration of IgG antibodies against BDC over time.* The patient population without pre-existing IgG specific for BDC are represented as a single line (—■—), the error bars represent S.D. Individual patients with pre-existing IgG against BDC are represented as follows; A047(◇), D008 (O), M027 (Δ), M129 (∇), S023 (▷), W007 (◁).

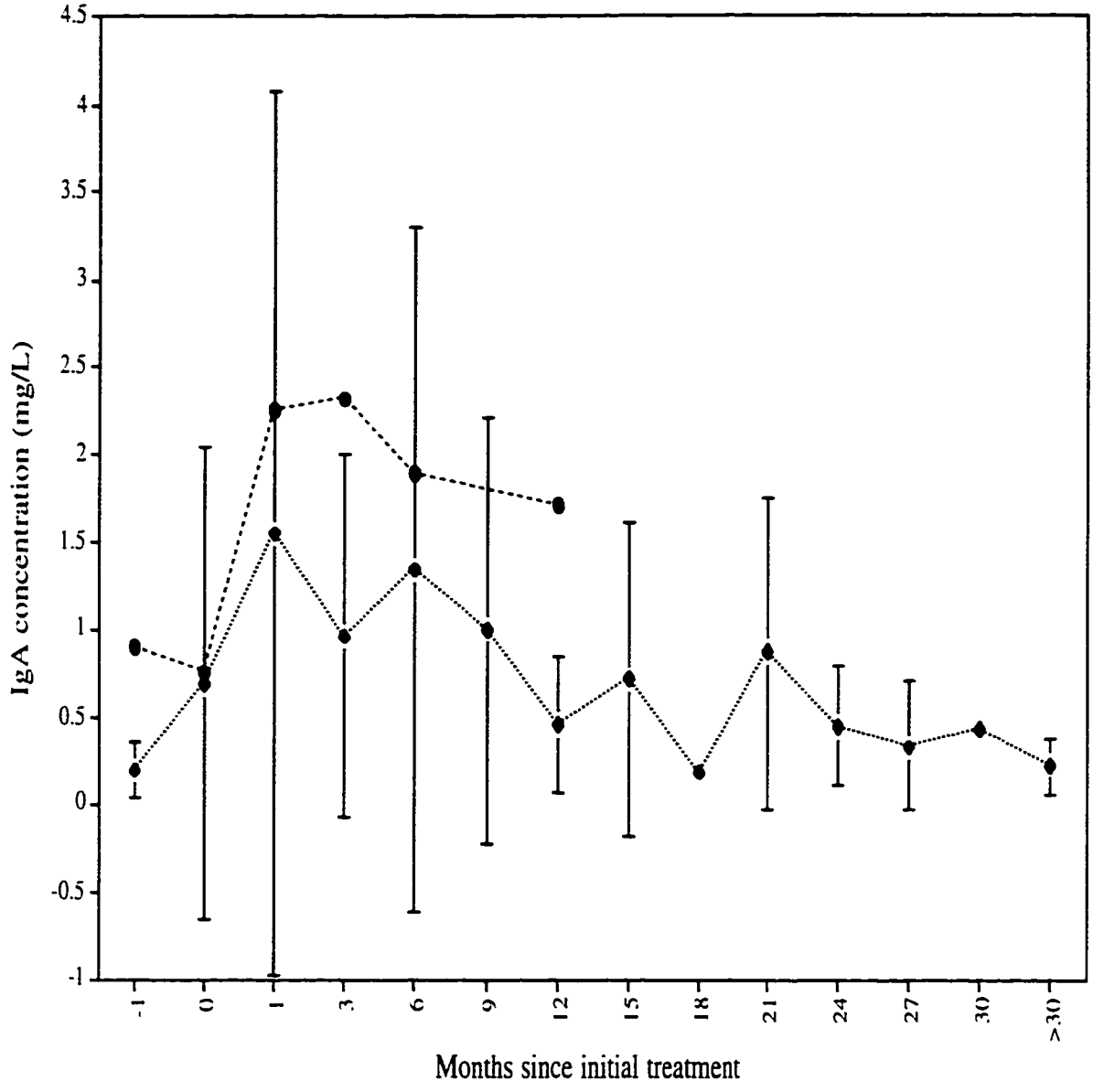

Fig 5. *The average concentration of IgA specific for BDC over time.* The patient (W007) with pre-existing IgA specific for BDC (—●—)is shown. The rest of the patient cohort (—◆—) , not exhibiting pre-existing IgA specific for BDC, is also shown with error bars representing 1 S.D.

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