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Primary Humoral Antibody Response to *Coxiella burnetii*, the Causative Agent of Q Fever

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Of 147 patients with acute Q fever diagnosed during a major outbreak in Birmingham, England, in early summer 1989, 41 provided sets of sera which allowed us to make a detailed analysis of the primary humoral immune response. Antibody titers specific for *Coxiella burnetii* were measured by the complement fixation test and by an immunoglobulin M (IgM)- and IgG-specific indirect immunofluorescence test. The relative avidity of specific IgGs was determined by the indirect immunofluorescence test with and without treatment of antigen-antibody complexes with 8 M urea. The IgG subclass responses after primary infection and their avidities were also determined for a limited number of paired serum specimens. Specific IgM titers persisted for more than 6 months in the majority of cases and were therefore not a sufficient criterion for the diagnosis of recent infection. However, for serial samples the antibody titer ratios (IgG/IgM) and the ratios (IgG titer with treatment/IgG titer without treatment) that indicated relative avidity changed significantly, depending on the time postinfection. Within the IgG class, the *C. burnetii*-specific antibody response over time was almost exclusively represented by subclass 1 molecules, which thus showed affinity maturation.

Q fever is caused by *Coxiella burnetii*, a member of the family *Rickettsiaceae*. The microorganism is endemic throughout the world and is mainly transmitted subclinically through animals (cattle, sheep, goats, rodents, cats, etc.). Particularly high titers of *C. burnetii* (10^9 infectious doses per g of tissue) are found in fetuses and placentas. The ability to form endosporelike structures may explain its resistance to drying and its ability to survive in aborted material for months. The infectivity for animals and humans is very high, with as low as one to five organisms being sufficient for infection. The usual way of transmission is by inhalation of infected aerosols (for a review, see reference 32).

Human Q fever outbreaks are frequently reported (6, 9, 12, 17, 21–24, 31), and the source of infection is mostly chronically infected animals (sheep, cattle) (1). Sporadic cases in humans in endemic areas have also been described (29).

In the early summer of 1989, a large Q fever outbreak occurred in the Birmingham, England, area (13, 24, 33) in which 147 people were diagnosed with acute Q fever. A fourfold rise in titer to the phase II antigen of *C. burnetii*, which was measured by the complement fixation test (CFT), or a sustained titer of greater than 1:256 by CFT were diagnostic of Q fever. The outbreak was probably caused by microorganisms that were produced and distributed during the lambing season (February to April), but the origin of the infection could not be firmly established. More details of the clinical features and the epidemiology of this outbreak have been reported elsewhere (13).

Complement-fixing antibodies against *C. burnetii* have been reported to arise during the second week after the onset of illness and to persist at high levels for many weeks (8, 18, 25). Specific immunoglobulin M (IgM) antibodies were found to be present from the second week onward and to persist for 10 to 17 weeks (5, 8). Here we describe some new observations of the humoral immune response to *C. burnetii* in a number of these patients. It was shown previously that the overall avidity of antibodies against specific antigens increases as the time after the onset of infection passes (7, 14, 19, 20). Not only have IgM antibodies, which constitute the major part of the antibody response during the first few weeks after primary infection, been shown to be of lower avidity than the IgG antibodies that occur predominantly in the later part of the infection (7, 14), but specific IgG antibodies to the specific antigens have also been demonstrated to increase in avidity with time (2, 14, 15, 30, 34). Furthermore, it has been observed that differences in the avidities of IgG molecules can be associated with different specific IgG subclass antibodies (3, 35). Mild detergents (guanidine hydrochloride [15], urea [30], and diethylamine [3, 35]) have been used in enzyme-linked immunosorbent assays to test for specific antibodies that have different avidities.

The determination of *C. burnetii*-specific IgM and IgG titers with (IgG + tr) and without (IgG − tr) 8 M urea treatment allowed us to draw conclusions with regard to the time of infection. The subclass specificity of the *C. burnetii*-specific IgG response was also investigated.

MATERIALS AND METHODS

CFT. Sera were heat inactivated (30 min at 56°C) and tested by CFT as described previously (11). As a control, serial serum dilutions were routinely tested in the absence of antigen in order to detect anticomplementary activity. *C. burnetii* phase II and I antigens were provided by the Division of Microbiological Reagents and Quality Control through the Public Health Laboratory Service. All serum samples obtained during the acute phase of the infection were tested against phase II antigen; serum samples taken 6

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TABLE 1. Dates of onset of illness and follow-up sera and CFT titers of sera from 27 pedigree cases of Q fever

Patient no.	Date (day/mo) of onset of illness in 1989	No. of days after onset of illness for:		CFT titers of follow-up sera (reciprocals of dilution)
		First serum (CFT, <1:8)	Follow-up serum (CFT, ≥1:32)	
1	24/4	7	14, 133, 187	512, 64, 64
2	27/4	7	64, 69, 210	128, 256, 256
3	27/4	6	15, 190	128, 512
4	27/4	13	20, 189	128, 1,024
5	28/4	11	19, 54, 110, 188	128, 128, 64, 16
6	28/4	10	17, 124, 188	256, 512, 256
7	30/4	8	25, 143, 186	512, 512, 256
8	2/5	9	79, 190	1,024, 512
9	3/5	6	22, 120, 190	512, 512, 512
10	4/5	4	39, 104, 185	64, 8, 8
11	6/5	6	18, 102, 194	256, 1,024, 512
12	7/5	3	12, 116, 120	512, 128, 64
13	7/5	4	15, 103, 186	512, 256, 128
14	9/5	6	14, 191	32, 32
15	11/5	4	76, 148, 182	256, 128, 128
16	11/5	5	14, 124, 202	128, 16, 16
17	13/5	6	16, 105, 194	1,024, 512, 256
18	13/5	5	10, 194	256, 512
19	15/5	4	13, 58, 126, 192	128, 1,024, 1,024, 256
20	15/5	4	17, 192	128, 16
21	15/5	3	23, 121, 192, 393	128, 16, 16, 8
22	17/5	5	26, 118, 193	512, 512, 256
23	17/5	5	14, 34, 125, 190, 294	32, 1,024, 1,024, 1,024, 256
24	18/5	3	47, 153, 182, 406	128, 64, 32, 16
25	19/5	6	13, 39, 167	128, 256, 512
26	21/5	7	12, 17, 186	256, 512, 64
27	27/6	7	13, 45, 150	128, 1,024, 256

months or later after the onset of illness were also screened against phase I antigen.

Indirect immunofluorescence test (IFT). Slides containing smears of formaldehyde-inactivated *C. burnetii* (phase II; Ninemile and Henzerling strains; BioMerieux, Lyon, France) were treated for 30 min at 37°C with serial dilutions of patient sera (which were checked previously for the absence of rheumatoid factor by using the Rheumatest kit produced by Wellcome, Beckenham, United Kingdom). This was followed by washing for two times for 5 min each time either in phosphate-buffered saline (PBS) or in PBS containing 8 M urea and by subsequent dipping in distilled water. Smears were then probed with fluorescein-conjugated sheep anti-human IgM or IgG antibodies, washed thoroughly, and examined by incident blue light fluorescence microscopy with a Zeiss microscope. The dilution that resulted in the staining of approximately 50% of the organisms was taken as the end point and defined the titer.

IgG subclass studies involved reaction of serial dilutions of patient sera with the *C. burnetii* smears and was followed by the addition of mouse monoclonal antibodies specifically directed to human IgG1, IgG2, IgG3, and IgG4 subclass epitopes (catalog nos. M09018, M10015, M08010 and M11013, respectively; Oxoid Unipath Ltd., Basingstoke, England). All monoclonal antibodies were used at a dilution of 1:100. Subsequently, slides were probed with fluorescein-conjugated sheep anti-mouse IgG. These studies were carried out with and without urea treatment as described above.

Statistics. Standard errors of arithmetic means of days after the onset of illness were calculated, and the significance of the difference of the means was obtained by using a *t* test. Calculations of linear regression and correlation coefficients were carried out by using the Oxstat program. For calculation purposes, titers of <8 (CFT) and <20 (IFT) were called 4 and 10, respectively, and titers of >512 (CFT) and >2,560 (IFT) were called 1,024 and 5,120, respectively.

RESULTS

The Q fever outbreak in the Birmingham area comprised 147 diagnosed cases, with the dates of onset ranging from early April to late June 1989 (13, 27, 30). Sequential serum samples from 41 patients with a known date of onset of illness were studied in more detail. From 27 of those patients we received a first serum sample which was negative for *C. burnetii*-specific complement-fixing antibodies within 2 weeks after the onset of illness (Table 1); two to five CFT-positive follow-up serum samples (a total of 80 serum samples) (Table 1) were collected from each of these "pedigree cases." From three patients we received CFT-negative sera at 20, 33, and 37 days after the onset of illness; this was followed by sera with high specific CFT titers. From the remaining 11 patients with a known date of onset of illness, we obtained serum samples which all showed high specific complement-fixing antibody titers; i.e., the date of seroconversion was missed.

For the 27 pedigree cases, the time of onset of clinical symptoms prior to the collection of the first serum sample was usually very short: 5.5 ± 2.4 days (range, 3 to 12 days). In 19 of the 27 pedigree cases, we had CFT-positive follow-up serum samples within 2 weeks after we received the first CFT-negative serum sample (Table 1). Evaluation of these data (data not shown) allowed us to conclude that a significant rise in CFT titer (from <1:8 to ≥1:32) was reached in 6.5 ± 3.5 days and that further rises (to titers of

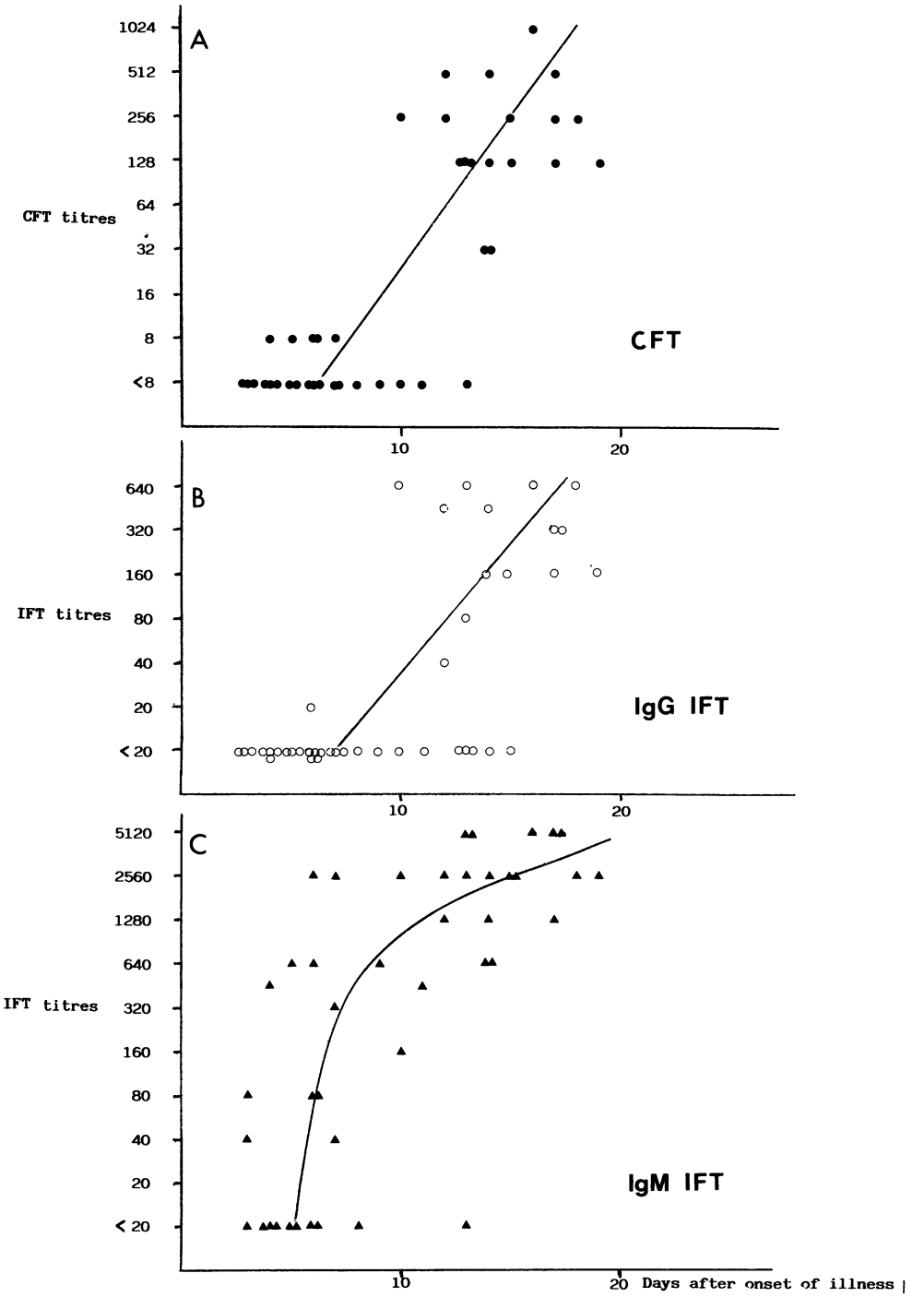

FIG. 1. *C. burnetii* titers measured by CFT (A), IgG IFT (B), and IgM IFT (C) in 42 serum samples obtained during the first 19 days after the onset of illness.

1:128 to 1:512) were reached within an additional 2 to 3 days. There was no clear relationship between the time of seroconversion after the onset of illness and the age of the patient (data not shown).

Thus, the following sequence of events emerged. The peak of onset of symptoms, according to the epidemic curve (13), was in the first week of May 1989, which dates the major event of infections back to approximately 10 April 1989, assuming a medium incubation period of 21 days (range, 14 to 28 days) (32). On average, significant CFT titers of ≥1:32 could be recorded 12 days (range, 8 to 20 days) after the onset of illness (sum of 5.5 ± 2.4 days between the onset of illness and the first negative serum sample plus 6.5 ± 3.5 days between the date of the negative serum sample and the date to reach a titer of ≥1:32).

The majority of CFT titers remained high over a period of 6 months (≥1:32 in 22 of 27 [81.5%] of cases; Table 1) and reached levels of between 1:8 and 1:256 after 10 to 14 months

TABLE 2. Emergence of *C. burnetii*-specific CFT, IFT IgG, and IFT IgM antibodies during the 20 days after the onset of illness

Time (days) after onset of illness	CFT titer					IFT IgG titer					IFT IgM titer				
	<8–16		≥32			<20–40		≥80			<20–40		≥80		
	Direct	Cumulative[a]	Direct	Cumulative	% Cumulative[b]	Direct	Cumulative[a]	Direct	Cumulative	% Cumulative[b]	Direct	Cumulative[a]	Direct	Cumulative	% Cumulative[b]
1–4	7	24	0	0	0	7	28	0	0	0	5	12	1	1	8
5–6	9	17	0	0	0	9	21	0	0	0	4	7	5	6	46
7–8	4	8	0	0	0	4	12	0	0	0	2	3	2	8	73
9–10	2	4	1	1	20	2	8	1	1	11	0	1	3	11	92
11–12	1	2	2	3	60	1	6	2	3	33	0	1	3	14	93
13–14	1	1	7	10	91	4	5	4	7	58	1	1	8	22	96
15–16	0	0	3	13	100	1	1	2	9	90	0	0	3	25	100
17–18	0	0	4	17	100	0	0	4	13	100	0	0	4	29	100
19–20	0	0	1	18	100	0	0	1	14	100	0	0	1	30	100
Total no. of sera	24		18			28		14			12		30		

[a] According to Reed and Muench (28).
[b] The 50 percent end points according to Reed and Muench (28) were as follows: 11.0 days for CFT titers, 12.8 days for IFT IgG titers, and 5.8 days for IFT IgM titers.

(Table 1). These data are in agreement with the results reported by Lennette et al. (18).

For 42 of the 44 serum samples obtained during the first 19 days after the onset of illness (Table 1), CFT titers and IgG- and IgM-specific IFT titers were determined in parallel and are represented in Fig. 1. Furthermore, when the sera were grouped by negative and borderline titers rather than by positive titers (CFT, <8 to 16 and ≥32, respectively; IgM and IgG, <20 to 40 and ≥80, respectively) (Table 2), depending on the time that elapsed after the onset of illness,

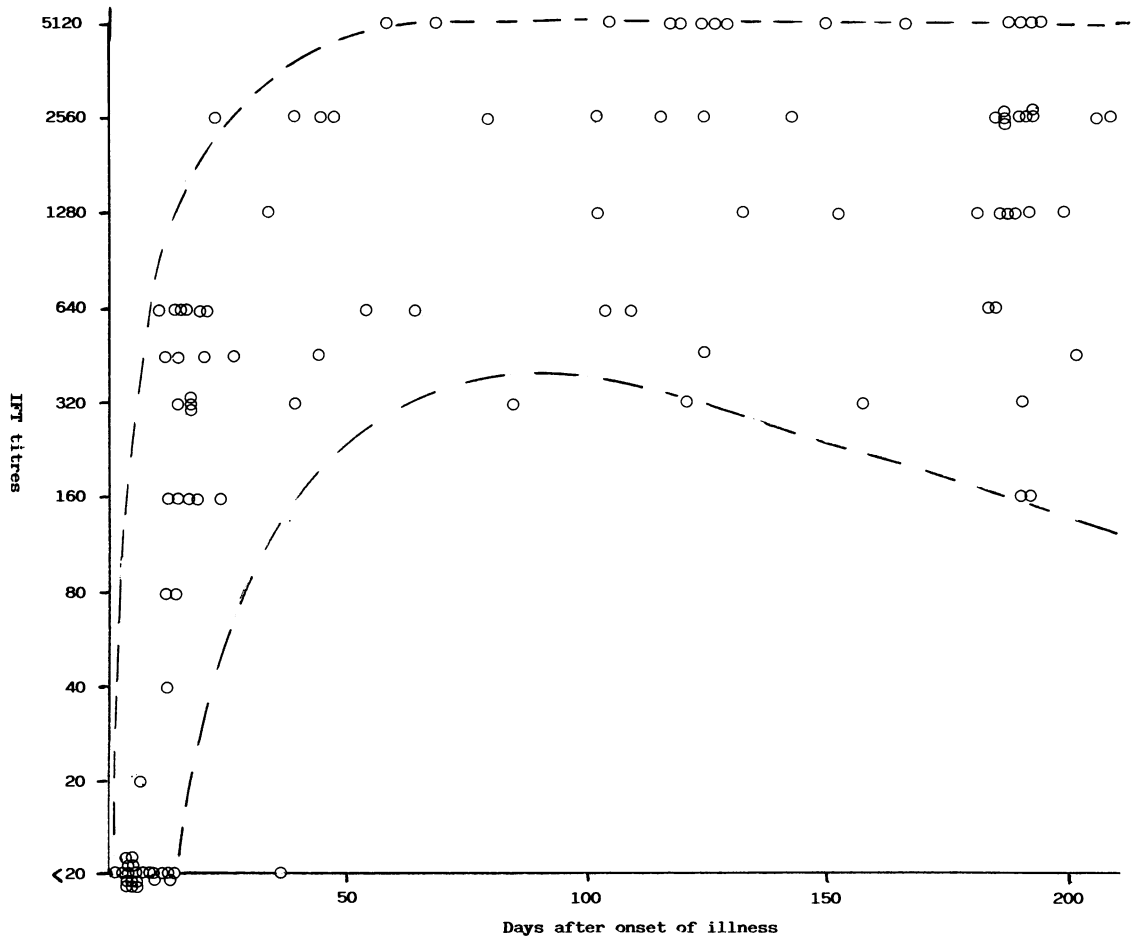

FIG. 2. IFT IgG titers against *C. burnetii* in patient sera obtained between 3 and 210 days after the onset of Q fever.

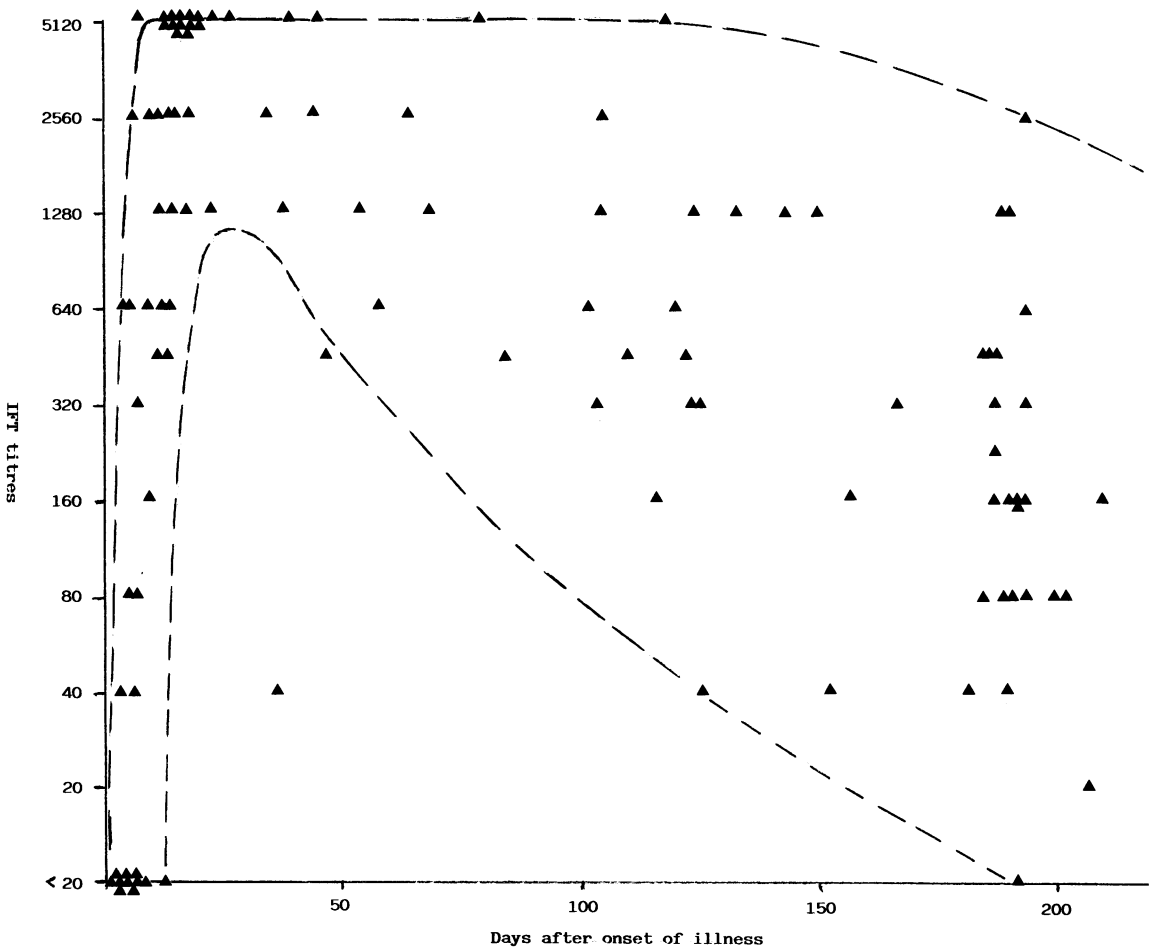

FIG. 3. IFT IgM titers against *C. burnetii* in patient sera obtained between 3 and 210 days after the onset of Q fever.

the following results were obtained. Whereas 50% of the serum samples had positive CFT and IgG IFT titers on days 11 and 13, respectively, 50% of the serum samples had IgM-positive IFT titers on day 6, i.e., 1 week earlier (Table 2). The data from Fig. 1 also suggest that CFT titers almost exclusively measure IgG molecules.

Follow-up studies up to 6 months after the onset of acute illness showed that IgG IFT titers were still significantly elevated in all cases (Fig. 2), whereas IgM IFT titers were elevated in 21 of 27 (77.8%) cases (Fig. 3). In three cases (Table 1), follow-up sera were obtained for 10 to 14 months; IgM antibodies disappeared from those serum samples, whereas the IgG titers were maintained at levels similar to those recorded 6 months after the onset of illness (data not shown).

Antibody measurements against phase I antigen in follow-up sera obtained after 6 months ($n = 90$, including sera from numerous patients for whom the exact date of the onset of illness was unknown) yielded a positive result in 17 serum samples (18.9%), but only in 1 case did the phase I/phase II titer ratio equal 1.

The 6-month follow-up serology for IgM antibodies (Fig. 3) indicated that their persistence made detection of IgM antibodies less than useful as a laboratory diagnostic criterion for a recent infection, and therefore, other means of evaluation were explored. Although specific IgM antibodies were still present in most follow-up serum samples at 6 months, their titers had generally fallen (Fig. 3). One possibility was to use the ratios (IgG/IgM) of the IFT titers and to plot them (as log values) against time after the onset of illness. This was done for 78 serum samples, and a significant line of linear regression was obtained (Fig. 4; $P < 0.0001$). From this it was deduced that the ratio of IgG/IgM titers is about 0.1 in the first 2 weeks after the onset of illness, approaches the value of 1 on approximately day 100 after the onset of illness, and climbs to over 10 during the following 100 days.

It was of great interest to discover that the ratios of IgG IFT titers with and without prior 8 M urea treatment of antigen-antibody complexes (see Materials and Methods) also seemed to follow a time course. When the (IgG + tr/IgG − tr) ratios were plotted against the day of onset of illness, the result was a straight line (Fig. 5) which was very similar to that obtained for the IgG/IgM ratios (Fig. 4) and which differed significantly ($P < 0.0001$) from 0. The ratios at the time of the IFT titer emergence were mostly between 0.02 and 0.1 and increased by a factor of 3 in approximately 100 days. The end point (reached in some but not all serum samples after 200 days) was, in this case, different because the (IgG + tr/IgG − tr) ratio could never surpass a value of 1. The slope of the log (IgG + tr/IgG − tr) ratios was about half as steep as that of the log (IgG/IgM) ratios when they were plotted against time after the onset of illness.

When the log (IgG + tr/IgG − tr) ratios were plotted

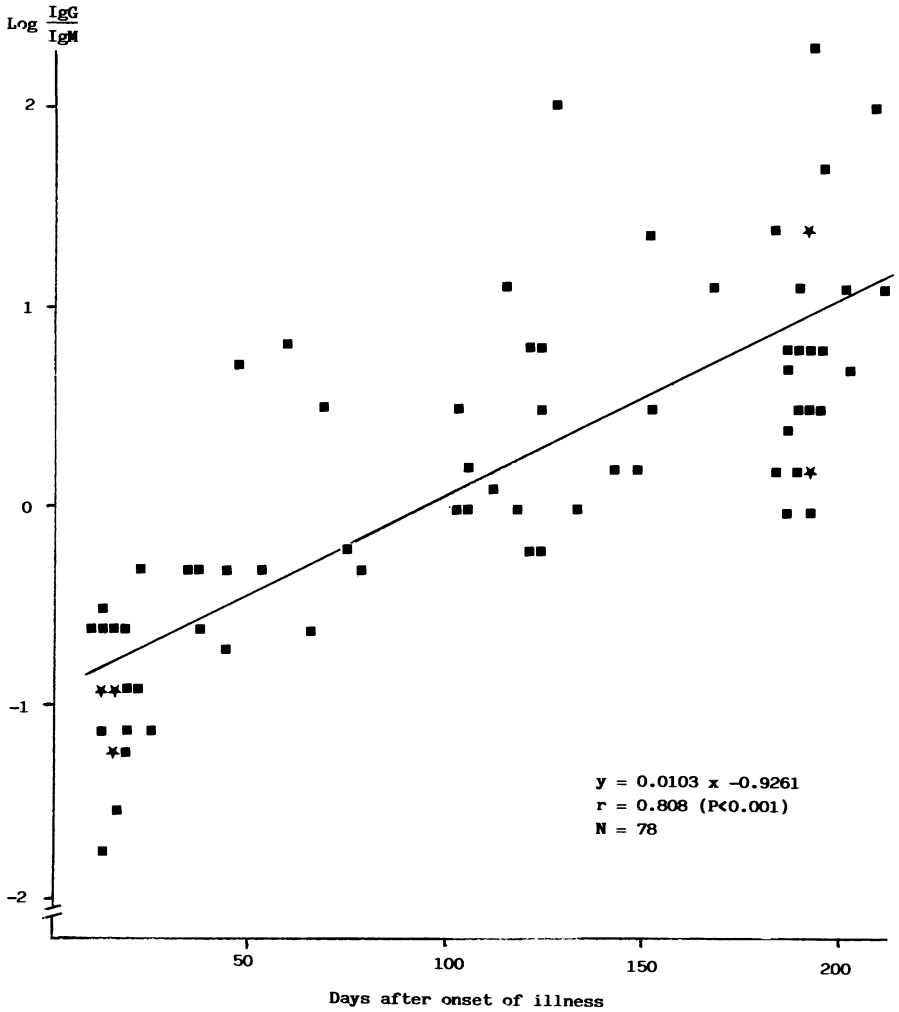

FIG. 4. Plot of log (IgG/IgM) values against time after the onset of Q fever. ■, one datum point; ★, more than one datum point.

against the log (IgG/IgM) ratios (Fig. 6), an excellent correlation of the ratios was found ($P < 0.0001$), which indicated that the two ratios followed the same time course.

The subclass specificity of the IgG response and the avidities of specific IgG subclass antibodies were investigated by using the IFT system. Whereas the total concentrations of IgG subclass molecules were within the normal ranges in all cases (data not shown), it was found (Table 3) that IgGs of subclass 1 (as well as IgM antibodies) form the major immune response following acute Q fever infection (Table 3) and that antibodies of this subclass initially are of low avidity. Studies on late-convalescent-phase sera indicate (Table 3) that IgG1 molecules remain the major IgG subclass involved and that they undergo an avidity maturation (4) with time.

DISCUSSION

During an outbreak of Q fever in Birmingham and its eastern conurbation area in the early summer of 1989 (13, 27, 33), 147 individuals were diagnosed as having acute infection. Sera from 41 patients were selected for further analysis according to the following criteria: (i) knowledge of the exact date of the onset of illness, (ii) availability of an acute-phase serum sample with a negative CFT titer against *C. burnetii* within 2 weeks of the onset of illness, (iii) availability of follow-up serum samples, the first of which showed a significant rise in titer (initially screened by CFT). These pedigree cases represented only approximately 28% of all cases but provided sufficient sera to allow a valuable analysis of the primary humoral immune response. The finding that, on average, only 5.5 ± 2.4 days elapsed before a serum which proved negative was taken and the observation that significant seroconversion took place 6 to 7 days later led us to the conclusion that at 12 days (range, 8 to 20 days) after the onset of illness, a significant CFT titer could be recorded. The IgG IFT titer was in exact concordance with the CFT titer (Fig. 3). This is in agreement with the data of Dupuis et al. (5); however, they found a significant average CFT titer for the first time after 20 days, i.e., approximately 8 days later than revealed by our data. Lennette et al. (18) recorded significant complement-fixing and agglutinating antibody titers in the majority of cases at the end of the second week after the onset of illness.

Significant IgM titers (≥80) were found on average on day 7 after the onset of illness, i.e., 1 week earlier than the first IgG titers became measurable (Table 2). This again agrees with the finding of Dupuis et al. (5). However, in our collection, IgM antibodies persisted at high titers for at least

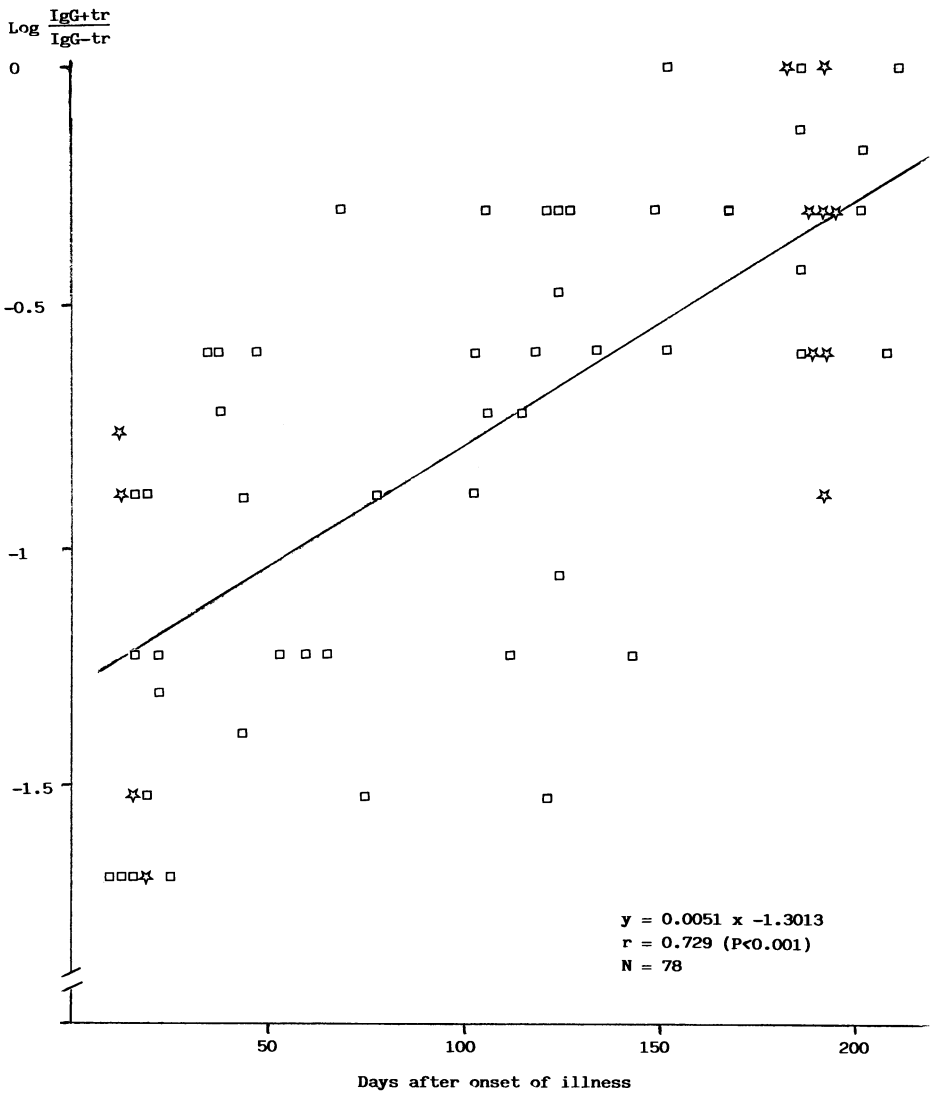

FIG. 5. Plot of log (IgG + tr/IgG − tr) values against time after the onset of Q fever. □, one datum point; ☆, more than one datum point; tr, treatment.

6 months in almost 80% of the cases (Fig. 3). In the study of Dupuis et al. (5), IgM antibodies were lost much earlier, and despite some caution in the discussion section of their report, Fig. 2 and the summary of their report seem to indicate that IgM antibodies were lost after 10 to 12 weeks. Field et al. (8) found that the longest period of persistence of specific IgM antibody was 17 weeks. Because this was not the case in our study (Fig. 3), the use of *C. burnetii*-specific IgM antibodies as a diagnostic criterion for recent infection was ruled out. Because specific IgM antibodies precede the appearance of specific IgG molecules, the presence only of IgM is compatible with a recent infection. However, such cases are rare, because laboratories usually receive serum samples from patients who are at a later stage of the illness. We therefore looked for other ways to determine the time of the onset of infection from the immune response in a single serum specimen.

Although specific IgM antibodies were still present in the majority of cases 6 months after infection, the IgM titers tended to fall. This prompted us to calculate IgG/IgM titer ratios which, when they were related (as log values) to the period that elapsed after the time of onset of illness, yielded a significant linear regression curve (Fig. 4) with a slope of approximately 0.01 and an intercept of −0.88. This means that the IgG/IgM ratio, which is, on average, 0.1 at the earliest time of detection of both antibodies, reached a value of 1 after 100 days and a value of 10 after 200 days. Given the subjectivity of reading IFT results and the variability of serological data as well as of the individual immune response, these calculations provide only rough guidelines (Fig. 4), but they are useful and sufficient for differentiating between infections in the recent (<3 months) and distant (>6 months) past.

It is known that the avidities of specific IgM antibodies, which are most prevalent early in infection, are low compared with those of IgG molecules, which are most prevalent later in infection (7). However, early specific IgG antibodies are also of lower avidity than late specific IgG antibodies, as previously shown for the immune response after rubella

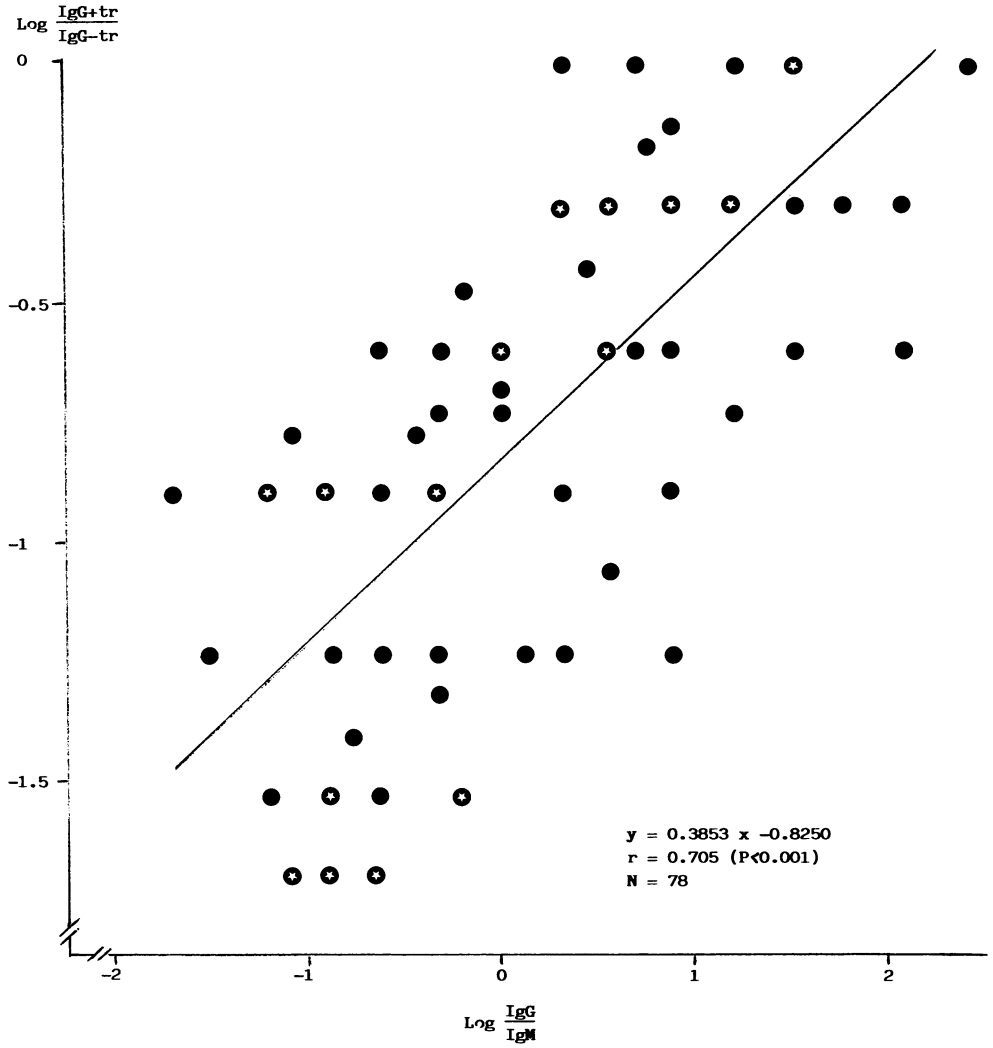

FIG. 6. Plot of log (IgG + tr/IgG − tr) against log (IgG/IgM) values. ●, one datum point; ✪, more than one datum point.

TABLE 3. *C. burnetii*-specific antibody titers in different IgG subclasses in early and late convalescent-phase serum specimens

Patient no.	Date (day/mo) of onset of illness in 1989	Time (days) after onset of illness	IFT titer[a] against *C. burnetii* measuring:									
			Total IgG		IgG1		IgG2		IgG3		IgG4	
			N[b]	tr[c]	N	tr	N	tr	N	tr	N	tr
2	27/4	64	640	40	200	<100	<100	<100	<100	<100	100	<100
		210	2,560	2,560	1,000	800	100	<100	<100	<100	200	<100
6	28/4	124	>2,560	480	>1,000	<100	100	<100	<100	<100	<100	<100
		189	2,560	1,280	800	600	<100	<100	<100	<100	<100	<100
28	29/3	99	1,280	<20	800	<100	<100	<100	100	<100	<100	<100
		216	2,560	2,560	800	600	<100	<100	200	<100	100	<100
11	6/5	102	2,560	640	>1,000	100	<100	<100	<100	<100	<100	<100
		189	2,560	1,280	1,000	400	<100	<100	<100	<100	100	<100
17	13/5	105	2,560	480	>1,000	<100	<100	<100	100	<100	<100	<100
		194	2,560	1,280	>1,000	800	100	<100	100	<100	<100	<100
19	15/5	58	2,560	320	>1,000	<200	<200	<200	<200	<200	<200	<200
		192	2,560	2,560	>1,000	1,000	200	<200	200	<200	<200	<200

[a] IFTs were used to measure the total IgG and IgG subclass-specific responses.
[b] N, the denaturant-free control (see text).
[c] tr, testing in the presence of 8 M urea during the washing steps.

virus infection (14, 15, 30). As a result, it was considered worthwhile to investigate the sera from patients with Q fever with regard to whether treatment of antigen-IgG complexes under mild denaturing conditions would reduce titers and to what extent this would depend on the time after infection when the serum was taken.

Indeed, we showed that early sera (collected within the first 3 weeks of onset) had specific IgG antibody which bonded with much less avidity (affinity) than IgG antibody of sera taken 100 days or later after the onset of illness. Thus, the titer ratio of (IgG + tr/IgG − tr) was initially low (under 0.1) and increased threefold within 100 days (Fig. 5); i.e., the increase was not as steep as for the IgG/IgM ratio. However, by setting up a single indirect IFT involving binding of serum to antigen in the presence and absence of denaturing agents (urea, diethylamine, guanidine [15, 30, 35]) that was followed in both reactions by detection with anti-human IgG-fluorescein conjugate (produced in sheep) and by comparing the resulting titers it was possible to provide data regarding the acuteness and/or recent occurrence of infection.

Because some data indicated that differences in avidity may be linked to certain IgG subclass responses (4, 26, 35), the IFTs were repeated as a double-sandwich test; antigen-IgG antibody complexes that formed in the presence or absence of urea were detected with anti-human IgG subclass-specific monoclonal antibodies. This was followed by detection with an anti-mouse IgG fluorescein conjugate. The results indicate that besides IgM, IgG molecules of subclass 1 represent the major component of the immune response. The IgG1 antibodies are also initially of low avidity, but later they become more avid (Table 3). No other IgG subclass was found to contribute significantly to the *C. burnetii*-specific primary immune response. The increase in the relative avidity of IgG1 antibodies during the primary immune response (affinity maturation according to Devey et al. [4]) is likely to be due to somatic hypermutation and clonal expansion of cells that produce antibodies within this subclass (10, 16).

In summary, we studied the primary humoral immune response against *C. burnetii* in a collection of well-characterized serum samples from 41 acutely ill patients. With increasing time from the onset of illness, specific high-avidity IgG1 subclass molecules replaced IgM and low-avidity IgG1 subclass antibodies. The low avidity of the primary IgG1 subclass response was found to be useful for diagnosing recent infection. Both titer ratios, IgG/IgM and (IgG + tr/IgG − tr), allowed us to distinguish between recent acute infections and those which occurred in the distant past.

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