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TITLE:

In vitro T-cell responses to β-lactam drugs in immediate and nonimmediate allergic reactions

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ABSTRACT

Background: β-Lactam drugs may induce both cellular and humoral allergic reactions, and there is evidence that T cells play an important role in the pathogenesis of these reactions. The aim of this work was to assess the sensitivity and specificity of the lymphocyte transformation test (LTT) as an *in vitro* diagnostic tool, in patients with either an immediate or a nonimmediate reaction to penicillin G and/or amoxicillin.

Methods: Fifty patients with a well-documented history of allergic reactions to β-lactams (31 immediate and 19 nonimmediate) were studied by means of skin tests (prick and intradermal), radioallergosorbent test (RAST), and, when necessary, controlled administration of the drug. Twenty-eight healthy subjects with good tolerance to penicillins served as controls. LTT was performed in all subjects.

Results: Skin tests were positive in 77.4% of the patients with immediate reactions and in 36.8% of those with nonimmediate reactions. The overall sensitivity of LTT in the allergic patients was 62%, but, when analyzed separately, sensitivity was 64.5% for the immediate group and 57.9% for the nonimmediate group. The LTT specificity was 92.8%.

Conclusions: The LTT should be considered a useful *in vitro* diagnostic tool to identify subjects allergic to penicillins, especially patients with nonimmediate reactions where the LTT has a better diagnostic value than skin tests. Interestingly, positive T-cell proliferative responses can be observed 10 or more years after the occurrence of the reaction without further exposure to the drug.

Key words: Drug allergy; β-lactams; lymphocyte transformation test; skin tests; T cells.

Introduction

β-lactams are still a major cause of immunologically mediated adverse drug reactions (1). According to the time interval between drug administration and the onset of symptoms, these reactions have generally been classified as immediate or non-immediate (2-5). Immediate reactions (IR) to β-lactams are well-defined clinical entities such as anaphylaxis and urticaria. They are known to be IgE-mediated although they need the collaboration of activated T cells that release cytokines, such as IL-4 and IL-13, which are necessary for the development of the humoral response (6). Although, there is *in vivo* and *in vitro* evidence of T lymphocyte involvement in the pathogenesis of non-immediate reactions (NIR) (3, 7) the underlying immune mechanisms implicated have not been fully elucidated. The presence in patients with NIR of activated peripheral blood T lymphocytes of both CD4 and CD8 phenotypes, with an increased expression of the cutaneous homing receptor (CLA), has recently been reported (8, 9). Moreover, mononuclear cells isolated from both peripheral blood and skin infiltrates from these patients can frequently be stimulated *in vitro* by the responsible penicillin (7, 10). In fact, several *in vitro* assays have been used to demonstrate lymphocyte sensitization to β-lactams, including the lymphocyte transformation test (LTT), MELISA (11), and generation of T cell lines and T cell clones (12, 13). The LTT has been studied extensively as an *in vitro* correlate of drug-induced cellular reactions with considerable disagreement among investigators (6, 14, 15). Basically, it consists of exposing peripheral blood mononuclear cells (PBMC) from sensitized individuals to the culprit drug of the allergic reaction in order to obtain *in vitro* evidence of lymphocyte proliferation. Various authors (16-18) have improved the method by using liver microsomal metabolites or *ex-vivo* generated putative metabolites, rather than the parent drug itself in the culture (19-21).

Although several studies have shown that LTT can identify allergic subjects, non-allergic exposed controls can also respond (14, 22), so that attempts to enhance the specificity of this T-cell proliferative response to drugs have been made, generating drug specific T-cell lines and clones from LTT cultures (12, 13).

The purpose of the present study was to evaluate the capacity of PBMC to respond *in vitro* to two relevant penicillin derivatives assessing the sensitivity and specificity of LTT as an *in vitro* diagnostic tool, in two well-differentiated groups of subjects allergic to β-lactams and a control group. Results are discussed in terms of the proliferative capacity and the usefulness of LTT for diagnostic purposes.

Material and methods

Patients

Fifty patients with a well-documented history of allergy to β-lactams confirmed by a positive skin test, RAST, or controlled administration of the drug were included in the study and were classified into two groups according to the time elapsed between administration of the drug and appearance of symptoms. All patients having urticaria/angioedema or anaphylaxis within 60 minutes of drug intake were included in Group A (IR). Criteria used to classify anaphylaxis or urticaria were those previously described (23). Those patients developing urticarial or exanthematic reactions more than six hours, but usually within 24-48 h, after β-lactam administration formed Group B (NIR). Nonatopic subjects with no history of adverse drug reaction and good tolerance to penicillin established by negativity of skin tests, RAST and controlled administration of oral amoxicillin, using the same methodology as for the patients, were selected as the control group (Group C). LTT was performed later, once the subjects had been defined as controls.

The study was approved by the Institutional Review Board, and informed consent was obtained from all the subjects before carrying out the study.

Skin testing

The determinants and maximum concentrations used were: benzylpenicilloyl poly-L-lysine (BPO) [$5x10^{-5}$ M] and minor determinant mixture (MDM) [$2x10^{-2}$ M], (both provided by Allergopharma Merck,Reinberck, Germany); amoxicillin (AX) [20 mg/ml], from Beecham Toledo, Spain; and ampicillin (AMP) [20 mg/ml] from Antibiotic S.A. León, Spain. Tests were all carried out as previously described (24, 25). Skin tests were done by prick, and if responses were negative, intradermal tests were performed.

Responses were classified as positive according to previously described criteria (26). In the prick tests, the sample was directly applied over the skin and a wheal bigger than 2 mm in two diameters with a negative response to the control saline was considered positive. In the intradermal test the wheal area was marked initially and 20 minutes after testing, and an increase in two diameters bigger than 3 mm was considered positive. In NIR, the erythematous induration area was evaluated 24, 48 and 72 hours after testing (27).

Quantification of specific IgE antibodies by RAST method

Specific IgE antibodies to benzylpenicilloyl-poly-L-lysine (BPO-PLL) and amoxicylloyl-poly-L-lysine (AXO-PLL) were determined as previously described (23). In brief, RASTs were performed in each serum sample in duplicate to BPO-PLL, AXO-PLL and PLL alone, and the results were expressed as the percentage of maximum radiolabelled anti-IgE uptake. The value given by PLL alone was then subtracted. Blood samples were drawn at the moment the patients were evaluated, and a serum sample was obtained and stored at –20ºC until assayed. All samples were made in parallel, and values were considered positive if they were higher than 2.5, which was the mean plus 2 SD of the negative control group. This system has been sufficiently validated, and the results published elsewhere (23).

Controlled administration

In order to establish the diagnosis and confirm the involvement of penicillin in the induction of the reaction, a controlled administration of the drug was made in those patients with negative skin test and RAST. Basically, this was carried out as described (24) with some modifications. In the first evaluation, if skin tests with BPO and MDM

were negative, we began with the parenteral administration of 1 ml of benzylpenicillin (BP) at 10^4 IU/ml, followed by 1 ml at 10^5 IU/ml and, if good tolerance was established at these doses, 1 ml at 10^6 IU/ml. Similarly, after performing the skin tests with AMP and AX, and if these were negative, AX was given by oral route at the following doses: 5, 50, 100, and 500 mg with a 1-hour interval between each. Controlled administration in controls was performed in all cases with oral AX.

Lymphocyte transformation test

PBMC were isolated from heparinized blood by ficoll density gradient centrifugation (Nycomed As, Oslo, Norway) from allergic patients and non-atopic controls. The LTT was performed as described (28), with minor modifications. Briefly, 2 × 10^5 PBMC/well were cultured in antibiotic-free RPMI-1640 medium (Bio Whittaker, Vervies, Belgium) supplemented with 2 mM L-glutamine (ICN Biomedicals Inc, Irvine, CA), 25 mM HEPES buffer (Seromed Biochrom KG, Berlin) and 10% heat-inactivated autologous serum in triplicate in 96 round-bottom microwell plates (Nunc, Roskilde, Denmark), in the presence of different drug concentrations: BP (Normon SA, Madrid, Spain) 1000, 500, 200 and 100 µg/ml, and AX (Beecham) 500, 200 and 100 µg/ml. Stock solutions of each drug were always freshly prepared immediately prior to use. Three well cultures without any antigen were used as controls to estimate the background proliferation. Tetanus toxoid at a concentration of 10 LF/ml (kindly provided by Berna Laboratories, Madrid, Spain) was used as a positive control for lymphocyte proliferation. The cultures were incubated for six days at 37ºC in 5% CO_2, and 18 hours before harvesting 1 µCi ^{3}H-thymidine (35 Ci/mmol, 1 mCi/1 ml; ICN Biomedicals Inc., Irvine, CA, USA) was pulsed to each well. The cultures were then harvested onto glass-fiber filters in a cell harvester (LKB Wallace, Turku, Finland) and the radionuclide

incorporation was measured by scintillation counting (Betaplate, LKB Wallace).

The stimulation index (SI) was calculated as the ratio between the mean values of counts per minute (cpm) obtained in cultures with antigen (drug) and those obtained in culture without antigen (background control). An SI ≥ 3.0 was regarded as a positive response.

Statistical Methods

The mean SI from the triplicate at the concentration giving maximal proliferative response was used for all calculations. Non-parametric values were compared by Kruskall-Wallis for the three groups. The SI was transformed to a log scale in the three groups and compared by ANOVA and the significance between two groups was determined by the Bonferroni test.

Sensitivity and specificity of the LTT was calculated as previously described (18) using skin tests, RAST and/or controlled administration of the drugs as the reference methods.

Results

Fifty patients with allergy to β-lactams were included in the study: 31 in Group A and 19 in Group B. In group A, there were 16 men and 15 women with a mean age of 46.6 ± 11.4 years (range 27-67), 29 of whom developed anaphylaxis and two urticaria. AX was reported to be the causative drug of the adverse reaction in 22 patients (70.9%), and BP in five (16.1%), and four patients (12.9%) were unable to specify which penicillin produced the reaction. In group B, there were three men and 16 women, with a mean age of 40.26 ± 14.32 years (range 19-63), 12 of whom developed non-immediate urticaria and seven exanthema, and again the drug most frequently involved was AX in 16 cases (84.2%), while three patients were unable to remember the culprit penicillin that induced the reaction. The control group (Group C) was formed by 28 subjects with skin tests and RAST negative to penicillins determinants and good tolerance to penicillin. There were 10 men and 18 women, with a mean age of 35.62 ± 12.25.

The immunological characteristics of Group A are described in Table 1. Twenty-four patients (77.41%) were skin test positive to at least one hapten, and 22 patients were RAST positive to at least one of the determinants tested (70.9%). All except four of these RAST positive patients were also skin test positive. The mean RAST value for BPO was 9.65±15.7 (range 0.1-65.1) and for AXO 7.82±14.1 (range 0.1-60.3). The mean time interval between the reaction and the performance of the tests was 892 days (SD 1943). In the three cases where skin tests and RAST were both negative, the diagnosis was confirmed by controlled administration of the drug. Patient 3A developed generalized urticaria 30 minutes after the administration of 100 mg of oral AX. Patient 18A developed palmo-plantar pruritus, dyspnea, conjuntivitis and generalised erythema 20 minutes after the administration of 1 ml of benzylpenicillin at 10^5 IU/ml. Patient 23A developed palmar pruritus, generalized erythema, conjunctivitis and dyspnea, 10 minutes

after the controlled administration of 100 mg of oral amoxicillin, and we had to administered epinephrine, steroids and antihistamines.

Table 2 shows the immunological characteristics of Group B. The mean time interval between the reaction and the study was 1836 days (SD 2604). Seven of the 19 patients (36.8%) presented non immediate intradermal reactions to AX and/or AMP. Skin test readings gave negative findings for BPO and MDM in all cases. All RAST values were negative for both BPO and AXO. The diagnosis in the 12 patients who were also skin-test-negative was confirmed by a controlled administration, showing, in all the cases, good tolerance to BP and positivity with the administration of oral AX. Eight of these subjects had an urticarial reaction between 24h and 48 h after AX intake, and four developed an exanthematic reaction, three of them 24-48h after administration of the drug and one with the onset of the exanthema beginning at 72h after challenge.

Tables 3, 4 and 5 show LTT results in groups A, B and C, respectively, and the time interval between the reaction and the performance of LTT. In group A (Table 3), six patients proliferated only in response to BP, eight exclusively to AX and six to both. In group B (Table 4), a positive response exclusively to BP was recorded in only one patient, while five patients responded selectively to AX and another five gave positive results for both BP and AX. In group C (Table 5), there was a positive response to BP in one control, and to AX in another. In the two cases with positive LTT responses, the penicillin was readministered later, and the subjects tolerated the drug, indicating that they were false positive in this assay. The mean SI from the triplicate of those concentrations of BP and AX giving the maximal proliferative response for each subject was used to calculate the mean values in the different patient and control groups. This mean SI value was 3.81±2.23 (range 0.9-7.7) in Group A, 7.88±8.07 (range 1.2-31.2) in Group B, and 1.80±0.83 (range 0.50-4.10) in the controls. The Kruskal-Wallis analysis and ANOVA

(using a logarithmic transformation of the data) showed statistical differences between the three groups ($p<0.001$). The Bonferroni test in the logarithmic data showed significant differences between Groups A and C ($p<0.05$) and Groups B and C ($p<0.001$) but no differences between Groups A and B ($p=0.2$). With a cut-off point of SI ≥ 3, the overall sensitivity of LTT in the allergic patients was 62%, but, when analyzed separately, sensitivity was 64.5% for the immediate group and 57.9% for the non-immediate group. The LTT specificity was 92.8%.

Discussion

β-lactams are the antibiotics most frequently inducing adverse reactions mediated by specific immunological mechanisms (29). In IR, subjects develop IgE antibodies that recognize either a common structure or a selective determinant of the β-lactam molecule (23, 30), and Th2 cells collaborate, enabling the cytokine microenvironment necessary for IgE isotype switching (31). Thus, as can be seen in the RAST and skin test results, in subjects with IR to AX two possibilities exist: either the patients present cross-reactivity with BP determinants, or they have selective side-chain specificity (31-35).

The recognition of β-lactams by T cells in subjects with NIR is also important, and experimental data support the participation of T cell responses in allergic reactions to these compounds (36, 37). Peripheral blood lymphocytes from patients allergic to penicillins have the ability to proliferate *in vitro* to the responsible drug (38-41), and β-lactam-specific T-cell lines and T-cell clones have been generated, with extensive descriptions of their cellular phenotypes and patterns of cytokine secretion (13, 28, 42-46). In addition, the presence of *in vivo* findings such as non-immediate skin rashes after controlled administration of the suspected drug (27, 37), and the evidence of a dermal infiltrate with a predominance of lymphocytes in skin biopsies (47) from lesional areas in patients with a NIR to β-lactams also lends support to T-cell involvement in these reactions.

The LTT is a well-established *in vitro* assay for detection of drug specific T-cell proliferation (13, 43), and, as an *in vitro* method, it has the advantage of being a safe procedure, avoiding possible sensitization or other adverse drug effects (6, 18)

The sensitivity of LTT to betalactams is quite variable, ranging from very low responses up to 100% in some studies (22). We have shown that PBMC from 62% of our patients proliferate *in vitro* in response to at least one of the penicillins tested,

independently of the type of reaction. This sensitivity is lower than that reported by Nyfeleyer and Pichler (14) in their series in 1997 (74%), but this can be explained by differences in patient selection, the SI cutoff levels chosen, and culture conditions. But, even though the sensitivity in our series was not as high as in others, we believe that LTT should be considered a useful *in vitro* diagnostic tool to identify subjects allergic to penicillins, especially those with non-immediate reactions, where it shows a better diagnostic value (57.9%) than skin tests (36.84%).

As mentioned earlier, T cells participate in both cell-mediated and humoral immune responses, so it is not surprising to obtain positive LTT responses in patients with an IR to penicillin, as has also been reported by others (13, 14). The sensitivity was higher for the IR group (64.5%) than for the NIR group (57.9%), but the proliferative responses in the IR group tended to be less intense (mean SI: 3.81) than in the NIR group (mean SI: 7.88), although these differences were not significant.

Our LTT results show heterogeneity in the antigenic determinant responses. Some of our patients showed specific responses to AX with good tolerance to BP, while other subjects responded to both. PBMC from patients in Group B were stimulated *in vitro* exclusively by BP in one case, by both BP and AX in five cases, and specifically by AX in five cases. This heterogeneity was even greater in the IR group, with six being stimulated with BP, six with both BP and AX, and eight with AX. These data support the idea that both the penicilloyl determinant and the side chain structure of AX play a role as nominal antigens, both contributing to the effective antigenic epitopes recognized by T cells isolated from penicillin-allergic individuals. In addition, our data also show that in allergic reactions to β-lactams there are no clear dose-response curves and the dose producing the highest proliferation varies from patient to patient.

It is of great interest that two of the non-atopic controls with established tolerance

to β-lactams were stimulated *in vitro* by BP in one case and AX in the other. This is a feature previously reported and not clearly explaines (14, 39). All controls in the study had been exposed to β-lactams occasionally in the past and had undergone the controlled administration of AX, and these positive *in vitro* responses may possibly be the result of T- cell sensitization and development of drug-specific immunologic memory, as others have suggested (42). But this clear discrepancy between *in vivo* and *in vitro* results could also be due to the different antigen concentrations used in both systems; thus, concentrations of β-lactams used in the LTT would be able to elicit and increase proliferative T cell responses that would not occur *in vivo* under normal circumstances (48). Whatever the case, our results show a more than acceptable LTT specificity (92.8%), confirming that LTT is a good diagnostic tool in allergies to β-lactams.

One of the most surprising findings in this work was that T-cell proliferative responses to β-lactams could be elicited ten years or more after the initial reaction occurred and the skin tests or controlled administration were performed, with no known re-exposure to the antigen in the intervening period. The transition from activated to resting memory T cells can be mediated through pathways not involving antigen recognition, although repeated antigen stimulation will result in an increase in the pool of cells that become memory. These memory cells must be long-lived and this could be achieved by two different mechanisms: either by generating a population of immortal non-dividing cells or by generating a dividing population that provides new cells as fast as old members are lost by cell death (49).

In summary, drug-allergic patients and healthy subjects have circulating memory T cells that can proliferate *in vitro* to β-lactam drugs. We need future studies aimed at determining the differences between the *in vivo* responses in patients and healthy subjects, and the mechanisms producing either the allergic reactions with clinical

symptoms in the case of the patients, or the lack of response in the case of non-allergic subjects.

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Table 1: Results of skin tests, RAST, and controlled administration of drugs in patients with Immediate Reactions.

	INT*	Drug	SKIN TESTS				RAST		CA
			BPO	MDM	AX	AMP	BPO	AXO	
1-A	3650	P	+	-	-	-	3.50	0.30	n.d.
2-A	2	AX	+	+	+	+	65.10	49.20	n.d.
3-A	4	AX	-	-	-	-	1.50	0.20	+ (AX)
4-A	1	AX	+	-	-	-	28.60	3.00	n.d.
5-A	30	AX	-	+	+	+	2.15	1.37	n.d.
6-A	1600	AX	-	-	+	+	0.10	5.33	n.d.
7-A	5	AX	-	-	-	-	0.01	5.10	n.d.
8-A	7	AX	-	-	+	+	36.60	60.30	n.d.
9-A	30	AX	-	-	+	-	0.97	3.53	n.d.
10-A	300	BP	+	-	-	-	16.12	4.44	n.d.
11-A	7200	BP	+	+	+	+	3.40	1.29	n.d.
12-A	720	P	-	-	-	-	9.60	6.20	n.d.
13-A	1	BP	+	+	-	-	37.60	0.20	n.d.
14-A	360	AX	-	-	+	-	0.14	0.78	n.d.
15-A	3	AX	-	+	+	+	0.09	0.64	n.d.
16-A	7	AX	-	-	+	+	0.38	24.02	n.d.
17-A	360	AX	-	-	-	-	0.01	2.50	n.d.
18-A	30	AX	-	-	-	-	0.25	0.71	+(BP)
19-A	4015	AX	-	-	-	-	2.10	2.60	n.d.
20-A	150	AX	-	-	+	+	0.60	3.50	n.d.
21-A	1080	P	+	-	-	-	20.00	3.55	n.d.
22-A	4	AX	-	-	+	+	1.82	20.96	n.d.
23-A	4	AX	-	-	-	-	0.63	0.01	+ (AX)
24-A	20	AX	-	-	+	+	0.01	17.70	n.d.
25-A	180	AX	-	-	+	-	1.20	0.59	n.d.
26-A	3	AX	-	+	+	+	0.09	0.64	n.d.
27-A	7200	BP	+	+	+	+	3.40	1.29	n.d.
28-A	360	AX	-	-	+	-	0.14	0.78	n.d.
29-A	1	BP	+	+	-	-	37.60	0.20	n.d.
30-A	300	P	+	-	-	-	16.12	4.44	n.d.
31-A	30	AX	-	-	+	+	7.30	17.70	n.d.

INT*: time elapsed between reaction and study in days; **Drug**: Causative drug of the adverse reaction. A**X**:amoxicillin; **BP**:benzylpenicillin; **P**: patients unable to specify which penicillin produced the reaction. **CA**: controlled administration. N.d.: not done. In skin tests, + means an immediate wheal reaction with that hapten.

Table 2: Results of skin tests, RAST, and controlled administration of the drugs in patients with non-immediate Reactions.

	INT *	Drug	SKIN TESTS				RAST		CA
			BPO	MDM	AX	AMP	BPO	AXO	
1-B	360	P	-	-	-	-	0.38	1.96	+ (AX)
2-B	730	AX	-	-	-	-	0.70	0.24	+ (AX)
3-B	720	AX	-	-	-	-	0.20	0.90	+ (AX)
4-B	365	AX	-	-	+	-	0.20	0.30	n.d.
5-B	2115	AX	-	-	+	+	0.01	0.10	n.d.
6-B	5040	AX	-	-	-	-	0.01	0.01	+ (AX)
7-B	210	AX	-	-	+	+	0.67	1.55	n.d.
8-B	1095	AX	-	-	-	-	0.30	0.55	+ (AX)
9-B	7	AX	-	-	+	+	0.50	0.58	n.d.
10-B	10220	AX	-	-	+	+	0.15	0.27	n.d.
11-B	120	P	-	-	+	+	0.00	0.00	n.d.
12-B	750	AX	-	-	-	-	0.30	0.40	+ (AX)
13-B	360	AX	-	-	-	-	0.10	0.20	+ (AX)
14-B	4656	AX	-	-	-	-	0.20	0.40	+ (AX)
15-B	3650	P	-	-	-	-	0.50	0.40	+ (AX)
16-B	120	AX	-	-	-	-	0.30	0.10	+ (AX)
17-B	3650	AX	-	-	+	+	0.60	0.50	n.d.
18-B	365	AX	-	-	-	-	0.70	1.10	+ (AX)
19-B	360	AX	-	-	-	-	0.10	0.20	+ (AX)

INT*: time elapsed between reaction and study in days. **Drug:** Causative drug of adverse reaction. **AX**: amoxicillin; **P**: patients unable to specify which penicillin produced the reaction. **CA**: controlled administration. N.d.: not done
In Skin tests: + means induration reaction with that hapten at 24-72h readings.

Table 3: Results from LTT, expressed as stimulation indexes, in patients with immediate reactions.

	Interval*	BP1000	BP500	BP200	BP100	AX500	AX200	AX100	TT
1-A	216	0.9	1.2	**5.6**	n.d.	1.1	1.3	n.d.	4.3
2-A	20	**4.7**	2.7	2.5	2.3	**7.4**	**6.0**	**5.4**	5.6
3-A	83	0.9	0.7	1.0	0.8	**4.9**	**3.5**	**4.5**	3.7
4-A	66	1.5	2.0	1.2	1.0	**3.2**	0.8	0.9	4.0
5-A	98	0.8	0.8	0.9	0.7	1.2	1.2	0.9	10.6
6-A	87	**3.0**	1.5	1.3	1.7	**3.3**	1.2	n.d.	3.8
7-A	25	0.6	1.1	0.9	1.1	0.5	0.6	1.3	9.4
8-A	11	1.3	1.2	1.3	1.4	1.1	1.2	1.0	1.6
9-A	80	1.6	1.5	**3.7**	**4.0**	**6.9**	**5.7**	1.1	5.6
10-A	71	**5.3**	2.5	2.4	2.5	1.5	1.2	1.4	4.3
11-A	346	0.6	0.5	0.7	0.6	**3.1**	0.5	1.0	10.9
12-A	38	**4.4**	**5.0**	**3.9**	**4.1**	2.0	2.7	2.3	8.2
13-A	9	**5.8**	**5.2**	**5.1**	**5.3**	**7.5**	1.9	2.1	3.3
14-A	102	0.5	1.0	1.0	0.9	0.8	0.4	0.7	3.1
15-A	110	0.6	0.4	1.1	1.2	0.7	0.7	1.0	2.8
16-A	54	0.9	1.7	0.7	0.9	1.1	1.1	1.3	8.0
17-A	45	1.9	1.9	1.5	1.6	**6.0**	1.1	1.3	3.2
18-A	55	2.5	2.4	2.1	1.3	**3.0**	2.2	2.0	3.6
19-A	9	0.8	0.6	2.5	1.8	1.1	2.0	1.7	99.9
20-A	13	0.5	0.6	0.7	0.9	0.7	0.7	0.7	1.1
21-A	192	0.8	0.9	1.4	1.6	1.0	0.6	0.5	28.7
22-A	84	2.5	**3.4**	1.7	1.6	1.6	1.6	1.0	45.0
23-A	144	0.4	1.7	0.9	0.9	1.5	0.9	0.8	5.2
24-A	72	2.3	1.9	1.7	1.6	**3.2**	2.7	1.1	3.7
25-A	102	**4.6**	**5.7**	**3.7**	2.2	**5.2**	**3.8**	2.8	15.5
26-A	132	2.1	2.0	2.0	2.0	2.3	1.8	1.0	7.2
27-A	372	2.2	2.6	1.7	1.6	**3.0**	2.6	2.6	13.0
28-A	120	**4.3**	**4.2**	**3.4**	**6.0**	**5.3**	**4.0**	**7.7**	16.1
29-A	36	**7.0**	**6.8**	2.3	2.0	2.6	2.0	1.9	12.5
30-A	106	**5.5**	**6.7**	**3.1**	2.6	0.8	0.9	0.8	13.1
31-A	61	2.2	2.4	1.9	1.2	**3.1**	2.7	2.5	9.6

*** Time interval:** time elapsed between reaction and LTT in months; **TT:** tetanus toxoid; stimulation index ≥3: positive response.

Table 4: Results from LTT, expressed as stimulation indexes, in patients with non-immediate reactions.

	Interval*	BP1000	BP 500	BP 200	BP100	AX 500	AX 200	AX 100	TT
1-B	137	1.0	1.1	1.0	1.3	1.6	1.5	1.1	7.8
2-B	11	0.5	1.1	1.0	0.7	1.2	0.7	1.1	3.1
3-B	164	**11.3**	**3.7**	**3.7**	**4.8**	**31.2**	**11.1**	**3.7**	6.6
4-B	22	1.8	1.4	1.6	1.5	**4.1**	**4.9**	**3.5**	20.8
5-B	91	0.9	0.8	0.7	0.9	1.7	2.9	2.3	3.1
6-B	240	2.1	2.3	1.4	1.6	**14.7**	**15.7**	2.2	9.4
7-B	51	2.4	2.3	2.1	2.0	1.0	0.9	1.1	9.0
8-B	37	0.5	0.6	1.3	1.4	1.2	1.4	0.5	7.4
9-B	1	2.6	2.5	**3.2**	2.6	**5.9**	**4.9**	**8.9**	6.0
10-B	346	1.0	1.8	1.1	1.0	0.7	0.6	0.8	99.9
11-B	129	**4.2**	**3.8**	**3.4**	2.2	**8.8**	**8.3**	**5.7**	21.5
12-B	48	1.6	2.7	2.4	2.5	2.7	1.5	1.6	3.4
13-B	154	**16.7**	**11.4**	**6.3**	**4.4**	**15.7**	**17.9**	**4.6**	4.2
14-B	238	0.9	1.4	1.7	2.1	1.6	1.4	1.3	6.9
15-B	240	**16.8**	**15.2**	**10.0**	**5.1**	1.0	1.3	0.9	3.3
16-B	20	1.4	1.0	1.7	1.4	2.9	1.1	**3.6**	73.2
17-B	196	0.8	1.5	**3.9**	**6.8**	**7.4**	**15.6**	2.0	99.9
18-B	14	2.4	1.8	1.2	1.5	2.1	**3.6**	**3.4**	3.8
19-B	180	1.2	1.1	1.0	0.8	**5.0**	**5.6**	**6.7**	11.0

*** Time interval:** time elapsed between reaction and study in months; **TT:** tetanus toxoid; stimulation index ≥3: positive response.

Table 5: Results from LTT, expressed as stimulation indexes, in controls.

	BP 1000	**BP 500**	**BP 200**	**BP100**	**AX 500**	**AX 200**	**AX 100**	**TT**
1-C	0.9	1.5	1.4	0.8	1.8	2.3	1.1	13.2
2-C	0.9	0.8	0.8	0.9	0.9	0.8	1.1	3.7
3-C	0.9	0.8	0.8	0.9	0.8	0.9	0.8	22.0
4-C	0.9	0.9	0.8	0.8	0.8	0.9	0.8	3.2
5-C	1.4	2.2	1.9	1.6	2.3	**3.1**	2.5	3.1
6-C	1.0	0.9	1.1	1.1	1.1	0.8	0.9	9.1
7-C	0.9	0.8	1.1	1.0	0.8	0.8	0.9	1.5
8-C	0.7	1.3	1.8	1.0	0.9	1.2	1.1	2.4
9-C	0.6	0.8	1.0	1.3	1.5	1.5	1.0	22.5
10-C	1.0	1.1	1.9	1.9	1.6	0.3	0.1	2.4
11-C	0.9	0.7	0.8	1.0	0.8	1.0	0.9	12.0
12-C	0.3	0.2	0.3	0.4	0.5	n.d	n.d.	2.9
13-C	1.0	1.6	2.0	1.4	1.7	1.9	2.7	34.5
14-C	0.5	0.4	1.1	0.8	0.4	0.5	0.2	3.3
15-C	1.3	0.6	0.7	0.9	1.1	1.0	0.9	10.0
16-C	2.2	2.3	1.6	1.7	0.4	0.9	0.7	2.5
17-C	1.9	1.3	0.7	0.7	0.5	0.8	2.2	14.3
18-C	0.9	1.4	1.3	1.0	1.3	1.9	1.4	22.2
19-C	0.9	1.6	1.5	1.5	1.5	1.6	2.8	3.6
20-C	0.7	**4.0**	**4.0**	**4.1**	1.2	0.7	0.9	16.9
21-C	0.9	1.7	1.3	0.7	1.3	1.2	2.0	5.2
22-C	0.7	0.8	1.0	1.7	1.6	0.4	0.5	4.1
23-C	1.0	0.8	1.3	1.0	2.7	1.3	1.8	27.2
24-C	0.7	0.6	0.6	0.8	1.6	0.8	0.9	8.4
25-C	0.7	0.6	0.5	1.1	0.9	1.5	0.5	4.7
26-C	1.8	1.8	1.7	1.2	1.0	2.8	2.4	9.5
27-C	0.9	0.9	0.9	0.8	1.4	1.6	1.0	13.3
28-C	0.7	0.6	0.5	0.5	1.0	0.7	0.6	4.7

n.d.: not done; TT: tetanus toxoid; stimulation index ≥3: positive response.