

Immunologic cross-reactivity between respiratory chemical sensitizers: Reactive dyes and cyanuric chloride

Meinir Jones, PhD,^a Cynthia Graham, PhD,^b Anthony Newman Taylor, FRCP, FFOM,^a Katherine Sarlo, PhD,^c Vivienne Hoyle, PhD,^d and Meryl H. Karol, PhD^b *London and Rusham Park, United Kingdom, Pittsburgh, Pa, and Cincinnati, Ohio*

Background: CyCl is a low molecular weight reactive chemical used as an intermediate in the production of plastics, herbicides, pharmaceuticals, and fiber-reactive dyes. It is a potent inducer of specific IgE antibody. The CyCl functionality is a structural component of monochlorotriazine and dichlorotriazine dyes.

Objective: We have investigated the immunologic cross-reactivity between cyanuric chloride (CyCl) and reactive dyes and it was hypothesized that this moiety might be a dye epitope and that it might stimulate an allergic antibody response in dye-exposed workers.

Methods: To test this hypothesis, we have used sera with IgE antibodies to CyCl and also sera from dye-exposed workers who have IgE antibodies to Procion Orange MX2R, an azo dye containing the dichlorotriazine group. As a control group we have used dye-exposed workers with IgE antibody to Remazol Black B, a diazo dye containing the vinyl sulfone-reactive group.

Results: Using RAST and RAST inhibitions, we identified negligible cross-reactivity between CyCl and dichlorotriazine dye.

Conclusion: The results of this study imply that the allergenic moiety on the dye residue resides in the chromophore rather than in the common structural component of CyCl and dichlorotriazine dyes. (*J Allergy Clin Immunol* 1998;102:835-40.)

Key words: Cyanuric chloride, reactive dyes, sensitizers, IgE antibody

Several low molecular weight reactive chemicals have been associated with occupational asthma and allergy.¹ One class of such chemicals contains fiber-reactive dyes.² The dyes contain a chromogen linked to a reactive group that enables the covalent attachment of the dye to hydroxyl groups on cellulose fibers. The chemical reactivity also allows the dyes to function as haptens and

Abbreviations used

CyCl: Cyanuric chloride

HSA: Human serum albumin

PrOr: Procion orange MX2R

Rem B: Remazol Black B

presumably to bind in vivo to carrier molecules and stimulate an immune response. The dyes that have been shown to cause occupational allergy and asthma in human subjects are predominantly the azo dyes or the anthraquinones.³ The reactive groups on these dyes are monochlorotriazines and dichlorotriazines, vinyl sulfone, bromoacrylamide, and monochlorodifluoro pyrimidine. Dye-specific IgE antibody has been detected in some workers exposed to these various dyes.

Cyanuric chloride (CyCl) is a reactive chemical that is used as an intermediate in the synthesis of plastics, herbicides, pharmaceuticals, and dyes.^{4,5} Specifically, CyCl is used to synthesize the monochlorotriazine or dichlorotriazine fiber-reactive dyes. It is an irritant and an allergen, with specific IgE antibodies detected in sera of exposed workers.⁶ In experimental systems, lymphocyte proliferation has been observed in lymph nodes of mice exposed topically to 2.5% to 10% of the chemical,⁷ and elevated levels of IL-4 and IL-10 have been detected in cultures of the lymph node cells, suggesting stimulation of T_{H2} cells.⁸ Therefore clinical and experimental data indicate CyCl can function as a hapten and stimulate immune responses. Because the CyCl functionality is a structural component of monochlorotriazine and dichlorotriazine azo dyes, we hypothesized that this moiety might be a dye epitope and stimulate allergic antibody in dye-exposed workers.

To test this hypothesis, we investigated serologic cross-reactivity between CyCl and reactive dyes. Human sera containing IgE antibody to CyCl were tested in RASTs and RAST inhibition studies with human serum albumin (HAS) conjugates of a panel of dyes, Remazol Black B (Rem B), a diazo dye containing the vinyl sulfone reactive group; Procion Orange MX2R (PrOr), an azo dye containing the dichlorotriazine group; and 2 other dichlorotriazine dyes with distinct chromophores.⁹ Additionally, reactivity of antibody from dye-exposed workers was tested with CyCl. Reactivity would be

From ^athe Department of Occupational and Environmental Medicine, Imperial College School of Medicine at the National Heart and Lung Institute, London; ^bthe Department of Occupational and Environmental Health, University of Pittsburgh, Pittsburgh; ^cThe Procter and Gamble Company, Cincinnati; and ^dThe Procter and Gamble Company, Rusham Park.

Received for publication Dec 23, 1997; revised June 11, 1998; accepted for publication July 14, 1998.

Reprint requests: Meinir Jones, Department of Occupational Medicine, Imperial College School of Medicine at the National Heart and Lung Institute, Manresa Rd, London, SW3 6LR, United Kingdom.

Copyright © 1998 by Mosby, Inc.

0091-6749/98 \$5.00 + 0 1/1/93062

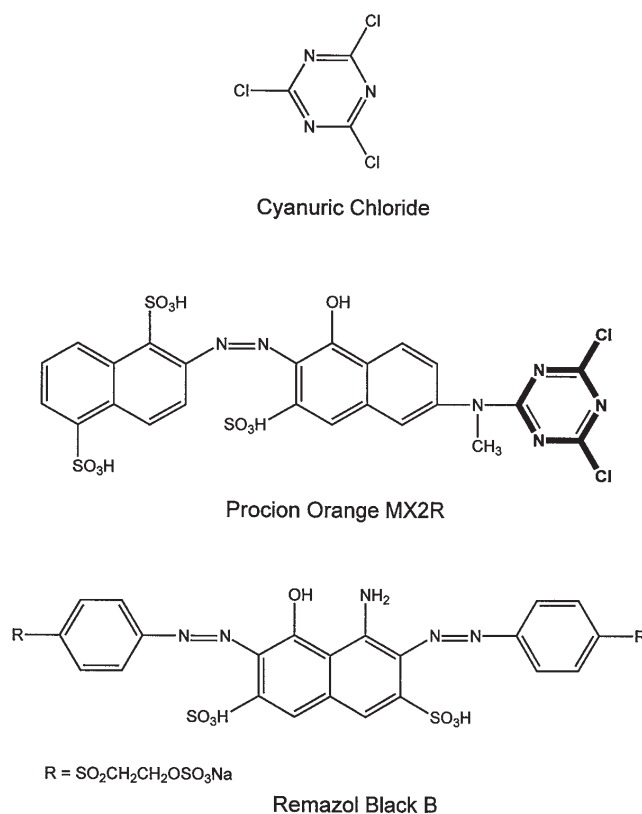


FIG 1. Structures of CyCl, PrOr, and Rem B.

expected if CyCl is an epitope of monochlorotriazine and dichlorotriazine dyes. Accordingly, cross-reactivity between CyCl and PrOr would be expected, but that between CyCl and Rem B would not. The results of this study indicate little cross-reactivity between CyCl and dichlorotriazine, implying that the allergenic epitope on the dye resides not in the dichlorotriazine moiety but rather in the chromophore. Inhibition of IgE binding to PrOr with dichlorotriazine dyes that have distinct chromophores also implies the allergenic epitope resides mainly in the chromophore.

METHODS

Antigens

CyCl-HSA was prepared as described.⁶ Briefly, CyCl (100%) was dissolved in acetone and added dropwise to a 0.5% solution of HSA (Sigma) in 0.05 mol/L phosphate buffer, pH 7.4. The molar ratio of reactants was 50:1. The haptenic content of the conjugate antigen, determined by using 2,4,6-trinitrobenzene sulfonic acid,⁶ indicated 46 mol CyCl bound per mol HSA.

HSA conjugate antigens with the dyes were prepared by separately dissolving 5 mg of PrOr (ICI), Procion Yellow MX4R (ICI), Ciba Yellow F3R (Ciba), and Rem B-vinyl sulfone (Sandoz) in 5 mL 2% HSA (in 50 mmol/L NaHCO₃ solution, pH 10.00). After 1 hour at 4°C, the solutions were dialyzed for 12 hours against PBS. The haptenic content of the PrOr conjugate was 19 mol PrOr per mol HSA.

TABLE I. RAST reactions of sera from CyCl-exposed workers

Sample	Total IgE (IU/mL)	HSA RAST (% bound)	CyCl-HSA RAST (% bound)	PrOr-HSA RAST (% bound)
1	84	0.3	20*	0.5
2	42	0.4	37*	2.8*
3	301	0.6	35*	2.8*
4	21	0.3	9*	0.3
5	135	0.5	7*	0.6
6	103	0.2	8*	0.4
7	252	0.6	15*	0.7
8	7	0.3	8*	0.3
9	78	0.3	13*	0.4
10	140	0.6	42*	5.2*

Values in bold indicate positive titer by regression method analysis.

*Positive titer by RAST ratio method.

Sera

Sera were obtained from 10 workers with occupational exposure to CyCl and from 11 dye-house workers who weighed and mixed dyes. The total IgE content of the sera was assessed by using RIA kits (Hycor, Ventrix Division, Portland, Me and Pharmacia Upjohn). Sera were stored at -70°C until use.

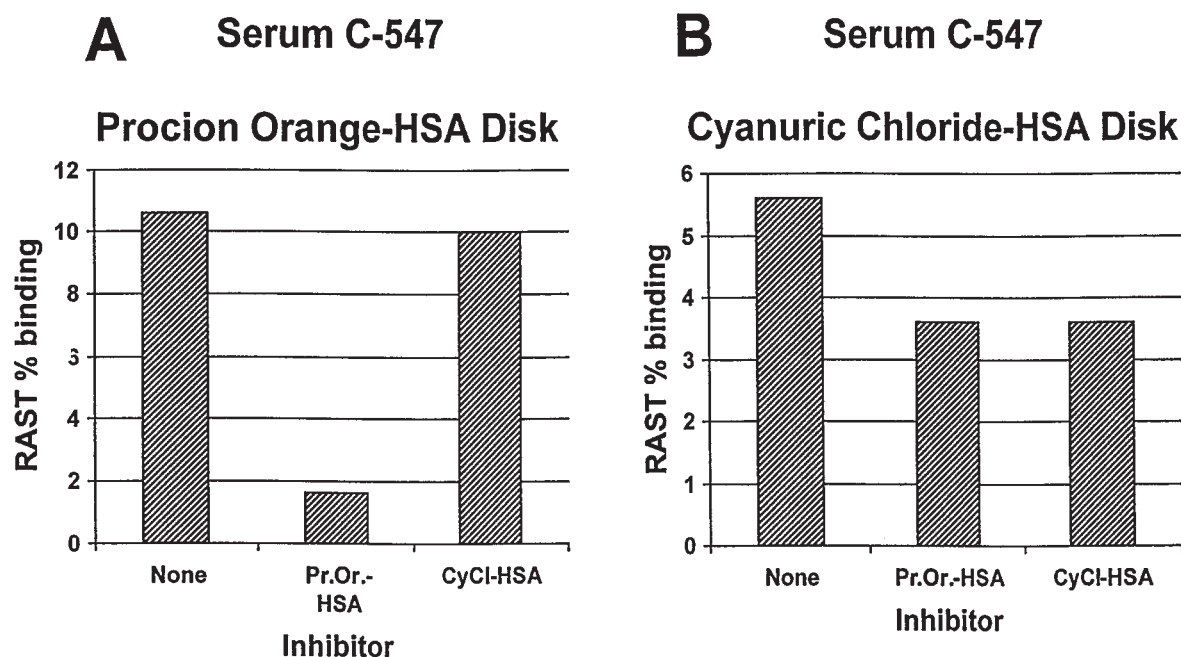


FIG 2. RAST and RAST inhibition of serum C547. **A**, Serum from this PrOr-exposed worker demonstrated 10.6% RAST binding to PrOr-HSA-coated disks that was inhibited by PrOr-HSA (1.6% binding), but not by CyCl-HSA (10.0% binding). **B**, Serum demonstrated 1.8% RAST binding to CyCl-HSA disks. Binding was inhibited equally by PrOr-HSA (0.7% binding) and by CyCl-HSA (0.9% binding).

TABLE II. RAST inhibition studies of CyCl-exposed workers

Sample	RAST (% cpm bound)					
	CyCl-HSA(d)	CyCl-HSA(d) CyCl-HSA(i)	CyCl-HSA(d) PrOr-HSA(i)	PrOr-HSA(d)	PrOr-HSA(d) PrOr-HSA(i)	PrOr-HSA(d) CyCl-HSA(i)
2	35	8 (76%)*	33 (6%)	2.3	ND	ND
3	50	14 (72%)	45 (9%)	3.5	ND	ND
10	71	19 (76%)	72 (0%)	6.4	1.4 (100%)	4.8 (43%)

Values in bold indicate significant inhibition of RAST binding.

d, Antigen-coated disk; i, inhibitor; ND, not done.

*Values in parentheses indicate the percent inhibition of binding.

RAST

Assays were performed in 2 laboratories. In each, antigen-coated disks were prepared by reaction of haptenated HSA with cyanogen bromide-activated paper disks.⁶ For the assay, a disk was added to a test tube containing 50 μ L of serum. After incubation at ambient temperature for 16 hours, the disk was washed and added to 50 μ L ¹²⁵I-anti-human IgE (Hycor-Ventrex and Pharmacia Upjohn). After 16 hours, the disk was washed and counted in a gamma scintillation counter. The amount of antigen-specific IgE antibody was expressed as the percentage of the added cpm that remained bound to the disk (percent binding). RAST positivity was assessed by 2 methods: the regression method described by Karol et al⁶ and the ratio method. In the ratio method a positive value is defined by the following equation:

$$\frac{\text{*Cpm hapten-HSA} - \text{cpm HSA}}{\text{HSA}} \geq 2$$

RAST inhibition assay

Hapten-HSA at 1 mg mL⁻¹ in PBS was mixed with an equal volume of serum. After 2 to 4 hours at 37°C, an antigen-coated disk was added, and assay was performed as described above. Inhibition of 25% or greater was scored as positive.⁶

$$\% \text{ inhibition} = \frac{100 (1 - \text{net cpm with inhibitor})}{(\text{net cpm with buffer})}$$

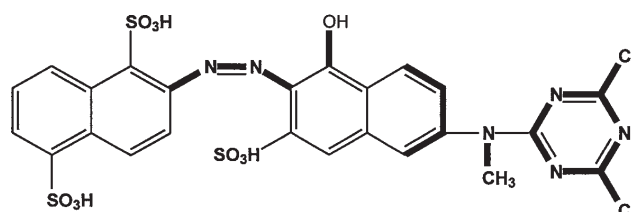
RESULTS

The structures of CyCl, PrOr, and Rem B are shown in Fig 1. Features of the CyCl structure found in PrOr dye are highlighted.

Sera from CyCl-exposed workers

Sera from 10 CyCl-exposed workers were selected on the basis of previous findings of positive CyCl-HSA

Structural Alerts Identified in Procion Orange MX2R



Structural Alerts:

Cl -C =
 OH -C =
 CH =CH -C =CH-
 SO₂-OH
 *N" -C=
 N -CH₃

The chemical has a high probability (>0.90) of being a respiratory sensitizer.

* Indicates N attached to a double bond.

FIG 3. Prediction of allergenic features of PrOr by using Structure Activity Relationship model.

TABLE III. RAST binding of sera from dye-exposed workers

Sample	IgE (IU/mL)	RAST (% cpm bound)				
		HSA	PrOr- HSA	Rem B- HSA	CyCl-HSA (RAST ratio)	CyCl-HSA (regression ratio)
C152	105	0.4	0.4	3.0*	0.4	ND
C156	42	0.4	0.7	3.1*	0.3	ND
C157	84	0.4	1.1	6.8*	0.6	3.2
C159	42	0.3	8.6*	1.7	0.3	3.3
C163	301	0.9	3.4*	0.7	1.5	5.6
C164	21	0.3	0.7	8.1*	0.5	2.8
C165	135	0.3	0.7	3.4*	0.6	4.0
C560	7	0.4	5.0*	5.1*	0.4	1.8
C561	78	0.3	8.0*	8.7*	1.1	3.9
C556	252	0.5	10.2*	0.7	1.2	4.1
C547	103	0.3	14.5*	1.7	2.0*	8.8

Values in bold indicate significant titer by the regression ratio method.

ND, Not done.

*Positive titer by RAST ratio.

RAST values.⁶ In the current study the sera were evaluated for binding to disks coated with CyCl-HSA, PrOr-HSA, and HSA alone. Results of the RAST assays are presented in Table I. Binding to CyCl-HSA disks ranged from 7% to 42% cpm bound, whereas that to HSA disks ranged from 0.2% to 0.6%. By using both methods of

RAST analysis, each of the sera was confirmed to remain highly positive for CyCl IgE antibody.

Assessment of IgE reactive with PrOr-HSA yielded much lower RAST values (Table I). Samples 2, 3, and 10 scored positive by using the RAST ratio method, although the absolute amounts of PrOr-HSA binding of

TABLE IV. RAST inhibition studies of IgE binding to PrOr in dye-exposed workers

Sample	RAST (% cpm bound)			
	PrOr(d)	PrOr(d)	PrOr(d)	PrOr(d)
		PrOr(i)	Procion yellow MX4R(i)	Ciba Yellow F3R (i)
C159	8.8	0.5 (94)*	4.6 (47)	5.2 (41)
C163	4.3	0.7 (84)	2.6 (39)	2.7 (37)
C560	4.1	0.5 (88)	4.1 (0)	4.3 (0)
C561	7.6	0.8 (89)	2.2 (71)	2.4 (68)
C556	7.0	0.6 (91)	8.2 (0)	6.7 (4)
C547	13.7	1.6 (88)	7.1 (48)	7.0 (49)

Values in bold indicate positive RAST binding of greater than 2% to the inhibitor.

d, Antigen-coated disk; i, inhibitor.

*Values in parentheses indicate the percent inhibition of binding.

samples 2 and 3 were very low. Only sample 10 scored positive by the regression method.

The specificities of the RAST reactions were evaluated by using RAST inhibition analyses. Serum 10 had exceptionally high binding (71%) to the CyCl-HSA disks (Table II). The binding was inhibited 73% by CyCl-HSA, but there was no inhibition with PrOr-HSA. Similarly, reactions of sera 2 and 3 with CyCl-HSA-coated disks were inhibited by CyCl-HSA, but not significantly by PrOr-HSA.

Testing was performed to determine possible reactivity of the antibodies in sera of CyCl-exposed workers with the dye antigens. As indicated in Table II, low levels of binding were obtained with PrOr-HSA-coated disks (column 5). Binding by serum 10 was significant and was inhibited completely by PrOr-HSA and by 43% with CyCl-HSA.

Sera from dye-exposed workers

Sera from 11 workers exposed to PrOr, Rem B, or both had been collected during 1985 to 1986 and stored at -80°C . The sera were thawed and evaluated for IgE antibody reactive with the dye conjugates, CyCl-HSA, and HSA. The CyCl-HSA RAST was performed in 2 laboratories and evaluated by the 2 methods. RAST results are provided in Table III. HSA RAST binding ranged from 0.3% to 0.9%. Samples C159, C556, C163, and C547 demonstrated IgE reactivity only with PrOr antigen. Samples C152, C156, C157, C164, and C165 were positive only in the Rem B-HSA RAST. Two samples, C560 and C561, were positive in both dye RAST assays.

Of particular interest in this study was the possible cross-reactivity of these sera with CyCl-HSA. One sample, C547, demonstrated such reactivity when assessed in both laboratories. C547 had CyCl-HSA RAST binding of 2.0% in laboratory A. Because the HSA RAST binding in this laboratory was 0.3%, the RAST ratio was positive. In laboratory B the serum binding was 8.8%, and total IgE was 103 IU/mL. These values resulted in a positive score in the RAST regression analysis.

The specificities of the RAST-reactive antibody serum C547 were examined by using RAST inhibition assays. Results are shown in Fig 2. Whereas antibody binding to PrOr-HSA-coated disks was substantial (10.6% cpm bound) and was inhibited to the extent of 84% by PrOr-HSA, CyCl-HSA did not inhibit the binding. By contrast, binding to CyCl-HSA-coated disks was low (5.6%) and was inhibited equally by CyCl-HSA and PrOr-HSA. Dyes with the dichlorotriazine functional group but with a different chromophore to PrOr did not consistently inhibit the binding of IgE to PrOr, and any inhibition was lower than that with the PrOr (Table IV).

DISCUSSION

Fiber-reactive dyes have been associated with cases of occupational asthma. The dichlorotriazine functionality on Procion dyes is responsible for its fiber reactivity by nucleophilic reaction with the hydroxyl groups on cellulose. The Rem B dye reacts by means of the B-sulphatoethyl sulfone group.⁹ Although CyCl has not been recognized widely as a cause of occupational asthma, we have previously reported detection of high titer of anti-CyCl IgE antibody in sera from workers occupationally exposed to the chemical.⁶ Because of the structural similarity of CyCl and the dichlorotriazine functionality on PrOr, we hypothesized that the latter functionality might be a major contributor to the allergenic activity of PrOr dyes.

A number of CyCl-exposed workers were found to have high titer of anti-CyCl IgE.⁶ Ten such sera were examined for possible cross-reactivity of these antibodies with PrOr dye. Results indicated that the dye was not able to inhibit the RAST reaction of any of the sera with CyCl-HSA, whereas CyCl-HSA was a very effective inhibitor in all cases (Table II). Furthermore, only 1 of the 10 sera gave a positive titer when tested with PrOr in RAST (Table I). The RAST titer was minimal (ie, only 12% of that obtained with CyCl-HSA). The reaction was inhibited more effectively by PrOr-HSA than by CyCl-HSA (Table II). These findings could be interpreted as either a minor cross-reaction of anti-CyCl antibodies or as independent production of antibody populations to each of the 2 allergens. Further analyses would be necessary to determine the correct explanation.

Sera were obtained from 11 dye-exposed workers, 6 individuals sensitized to PrOr dye and, for control purposes, 5 workers sensitized to an unrelated fiber-reactive dye (Rem B). Only one of the 11 sera (number 547) showed reaction in CyCl-HSA RAST. This serum was from a worker exposed to PrOr dye. The RAST titer to PrOr antigen was 6-fold greater than that to CyCl antigen. RAST inhibition studies indicated that only PrOr antigen was able to inhibit the binding to PrOr-HSA disks. The binding to CyCl-HSA disks was inhibited equally by PrOr and CyCl antigens, but not by Rem B-HSA. The latter finding excluded the possibility that binding was due to antibody recognition of HSA.

Contamination of PrOr dye with CyCl was considered as a possible explanation for the reaction of sample C547 with CyCl-HSA. However, the dye was known to be free

of organic contaminants. Moreover, if PrOr had been contaminated with CyCl, one would have expected positive PrOr RAST values from the CyCl sera. As evident from Table I, although these sera had very high titer to CyCl-HSA with only 1 exception, they did not react in PrOr RAST. Additionally, reactions with CyCl-HSA-coated disks were not inhibited with PrOr antigen (Table II). We therefore concluded that contamination of PrOr by CyCl was not a plausible explanation of the CyCl-HSA RAST binding of serum sample C547. Rather, a more likely explanation is that in this individual the antibodies to PrOr have some limited reactivity with the CyCl moiety.

Because only 1 of the sera from PrOr-exposed workers gave a positive result in CyCl-HSA RAST and dyes with the dichlorotriazine group but with a different chromophore did not consistently inhibit the binding of IgE to PrOr, it is unlikely that the dichlorotriazine functionality is a major allergenic epitope of the PrOr dye. We were able to obtain a prediction of the structural features of the dye that likely contributes toward respiratory allergenicity from our structure-activity model¹⁰ of respiratory sensitizers. The model identified several structural alerts as indicated in Fig 3. Portions of the chromophore, as well as of the dichlorotriazine moiety, were identified. This result indicates that areas outside the reactive portion of the dye likely contribute to the allergenic epitope.

In summary, a serologic study was undertaken to determine whether a common structural feature contributed to

the allergenicity of CyCl and PrOr dye. Results indicated that in all cases the common structural component was not the major allergenic epitope of the dye.

We thank D. Stoliker for secretarial assistance.

REFERENCES

1. Chan-Yeung M, Lam S. Occupational asthma. *Am Rev Respir Dis* 1986;133:686-703.
2. Docker A, Wattie JM, Topping MD, Luczynska CM, Newman Taylor AJ, Pickering CA, et al. Clinical and immunological investigation of respiratory disease in workers using reactive dyes. *Br J Ind Med* 1987;44:534-41.
3. Romano C, Sulotto F, Pavan I, Chiesa A, Scansetti G. A new case of occupational asthma from reactive dyes with severe anaphylactic response to the specific challenge. *Am J Ind Med* 1992;21:209-16.
4. Rydzynski K, Jedrychowski R. Sensory irritating properties of cyanuric chloride as revealed with plethysmographic method. *Int J Occup Med Environ Health* 1994;7:149-54.
5. Smolin EM, Rapport L. S-Triazines and derivatives. In: Weissberger A, editor. *The chemistry of heterocyclic compounds*. New York: Interscience, Inc; 1959. p. 116.
6. Karol MH, Kramarik JA, Ferguson J. Methods to assess RAST results in patients exposed to chemical allergens. *Allergy* 1995;50:48-54.
7. Kimber I, Weisenberger C. A murine local lymph node assay for the identification of contact allergens: assay development and results of an initial validation study. *Arch Toxicol* 1989;63:274-82.
8. Dearman RJ, Smith S, Basketter DA, Kimber I. Classification of chemical allergens according to cytokine secretion profiles of murine lymph node cells. *J Appl Toxicol* 1996;17:53-62.
9. Luczynska CM, Topping MD. Specific IgE antibodies to reactive dye albumin conjugates. *J Immunol Methods* 1986;95:177-86.
10. Graham C, Rosenkranz HS, Karol MH. Structure-activity model of chemicals that cause human respiratory sensitization. *Regul Toxicol Pharmacol* 1997;26:296-306.